BETACYANINS FROM *HYLOCEREUS UNDATUS* AS NATURAL FOOD COLORANTS

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SUMMARY

Hylocereus undatus is an epiphytic cacti with brightly colored fruits. These fruits, known by various names such as Dragon fruit, Pitaya or Strawberry cactus are harvested as a food crop in Vietnam, Australia and in South and Central America from where it originated from. The fruit peel is known to contain betacyanins, naturally occurring red pigments that are known to be non-toxic. In this project, the feasibility of employing the fruit peel as a source of natural colorants for coloring foods is investigated. Compared with betacyanins from beet root, currently the only commercially available betacyanin-based natural food colorant, betacyanins from *Hylocereus undatus* imported from Vietnam was found to be capable of superior shelf life even in the absence of refrigeration. Stability under commonly encountered food processing conditions was also demonstrated with the exception of exposure to elevated temperatures. Therefore, while the scope of application of these pigments remained confined to foods that experience minimal heat processing *e.g.* ice-cream, fizzy drinks *etc*, favorable properties for longer and less costly storage was demonstrated. Immense potential as such, exists, for the development of commercial natural food colorants derived from such pigments.

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ABTS: 2,2-Azino-di(3-ethylbenzthiazoline)sulfonic acid

BA: Betalamic acid

BetX: Betacyanin extracts from the fruit peel of *Hylocereues undatus*

CIE: Commission Internationale de L'Eclairage
(International comission on illumination)

CE: Capillary electrophoresis

CDG: Cyclodopa glucoside

DI: Deionized water

EU: European union

FDA: Food and Drug adminstration (USA)

HPLC: High performance liquid chromatography

LC: Liquid chromatography

MS: Mass spectrometry

1. INTRODUCTION

1.1 THE ROLE OF FOOD COLORANTS

"The best food with a perfect balance of nutrients is useless if not consumed.

Consequently, food needs to be attractive."

B.S. Henry[1]

The importance of aesthetic value and thus, appreciation of food is evident in the above quote. A British manufacturer reportedly suffered a drop of 50% in product sales after he omitted colours from his products, in response to public outcry against synthetic colours – the pre-dominant form of food colorants.[2]

As such, the appearance of foods and their taste (flavour) are crucial factors in their acceptance and appreciation. The paramount importance of the former is evident in several studies…… when foods are coloured so that the color and flavour are matched, for instance, yellow to lemon, green to lime, the flavour is correctly identified on most occasions. Identification is frequently erroneous, however, when the color, does not correspond to flavour (DuBose, 1980)[3]. Red drinks were also perceived to be sweeter than identical drinks that were either colourless or another color. (Pangborn, 1960)[4].

The appearance of food is closely related to its color. As differences in color are readily perceived, it is reasonable to suggest that color is of paramount importance where the appearance of food is concerned[5].

Hence, the following functions may be effected by food colorants:

(I) To reinforce colours already present in food but less intense than the consumer would expect.

(II) To ensure uniformity of color in food from batch to batch.

(III) To restore the original appearance of food whose color has been affected by processing.

(IV) To impart color to certain foods such as sugar confectionary, ice lollies and soft drinks which would, otherwise, be virtually colourless.

Colorant compounds are introduced into foods via a number of suitable application forms for instance, solutions based upon safe-to-consume solvents such as water and citrus oil. It is necessary that such compounds should exhibit adequate stability in the pH range of most foods (3 to 7) and good microbiological quality especially in the case of water-soluble and/or sugar containing compounds (*e.g.* anthocyanins). Inherent stability to elevated temperatures would be an added advantage[1].

During the first half of the 20th century, a large proportion of the colourings employed in the food industry were based on synthetic (azo-) dyes derived from coal tars. Natural colorings were far less common until relatively recently as a result of misguided notions that they were of poor tincture strength[6].

1.2 NATURAL FOOD COLORANTS

Prior to the 20th century, food colorings were derived from natural, mineral-based sources that were often dangerous. For instance, poisonous copper(II) sulphate was once used to color pickles, alum to whiten bread and cheeses were coloured with red lead, vermillion or mercury sulphate. With the imposition of the much needed food regulations in the United States in 1960, the food industry gradually turned to azo-dyes (Fig 1.1) as their main source of colorants[7,8].

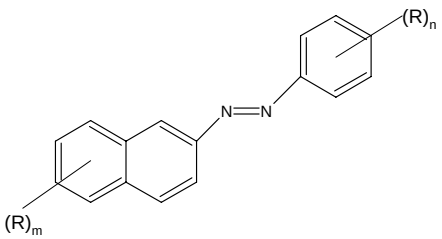


Fig 1.1 Representative structure of azo-dyes.

Azo-dyes are synthetic dyes *i.e*. they do not occur in nature but are generated via chemical syntheses. Hence, dyes of high purity and uniformity may be obtained. In addition, such dyes are brightly coloured and it is possible to obtain a full spectrum of colours by introducing selected functional groups e.g. $–SO_3^{2-}$, $-CH_3$, $-OCH_3$ into the basic azo-dye structure (Fig 1.2). As such, azo-dyes became popular with consumers for many years especially since signs of possible and/or carcinogenicity was not detected until recently[7].

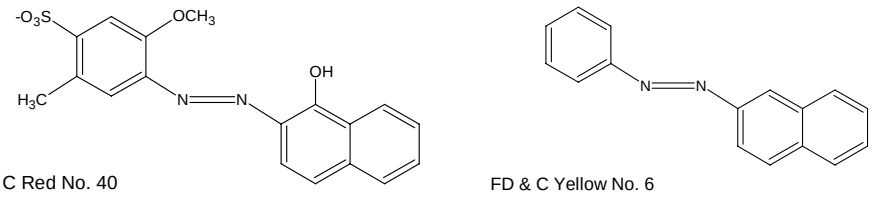


Fig 1.2 Examples of azo-dyes employed as food colorants.

The recent discovery of enzymes capable of azo-reductase activity in the small intestines, however, has raised safety concerns as regards the use of azo-dyes as food colorants[9]. This is in view of the fact that reduction of the azo linkage results in the formation of hydrazines[10] that undergo homolytic N-N fission readily to generate radicals which are potent carcinogens.

Such issues have culminated in stronger consumer preference for natural colourings in the form of organic pigments that are perceived by most, to be benign[11]. Thus, natural food colourants have been regarded and defined as organic pigments

that are derived from natural sources or concentrated extracts of these materials using recognized food preparation and/or extraction procedures. This definition, however, precludes caramels manufactured using ammonia and its salts and copper chlorophyllins, since both of these products involve chemical modification during processing[1].

Pigments are defined as chemical compounds that absorb light in the wavelength range of the visible region. The color produced is due to a molecule-specific structure (chromophore); this structure captures energy from photons resulting in the excitation of an electron from an external orbital to a higher orbital; the non-absorbed energy is reflected and/or refracted to be captured by the eye, and the generated neural impulses are transmitted to the brain where they are interpreted as a color.

Pigments are present in many organisms in the world but plants are the principal producers of such compounds. They may be present in leaves, fruits, vegetables and flowers. In addition, colors may also be found in skin, eyes and other animal structures and in microorganisms like bacteria and fungi. Apart from their inherent beauty, pigments have many other reported functions that include anti-cancer activity (*e.g.* betalains), UV protection (*e.g.* melanin), photosynthesis (*e.g.* chlorophylls) and in oxygen transportation (*e.g.* haemoglobin). There are two general methods to classify natural pigments. The first method classifies pigments based on the molecular structure of the chromophore. In this method, pigments are classified either as chromophores with conjugated systems (*E.g.* carotenoids, anthocyanins, betalains) or metal co-ordinated porphyrins (*E.g.* chlorophyll, myoglobin etc)[12]. The second

method[12] classifies pigments in accordance to their structural class as shown in table 1 on the following page.

Class	Examples
Tetrapyrrole derivatives	Chlorophyll and Haem colours
Isoprenoid derivatives	Carotenoids and Iridoids
N-heterocyclic compounds different from tetrapyrroles	Purines, Pterines, Flavins, Phenazines, Phenoxazines and Betalains
Benzopyrans derivatives (oxygenated heterocyclic compounds)	Anthocyanins and Flavonoid pigments
Quinones	Benzoquinone, naphthoquinone and anthraquinones
Melanins	

Table 1 Classification of naturally-occurring pigments in accordance to structural class

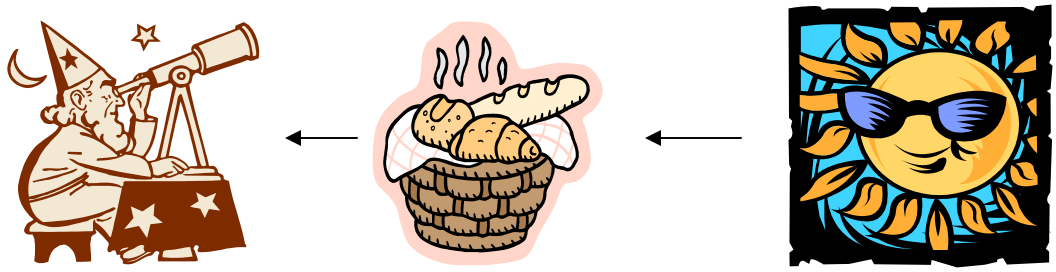
Therefore, while the number of pigments in the world is extremely large, the number of pigment categories is surprisingly small. Their general characteristics include susceptibility to heat, extreme pH, oxygen and strong light. These represent inherent weaknesses of natural pigments as food colourings. Their applications are thus, limited to foods that receive minimal heat processing e.g. jellies, ice-cream, fizzy drinks etc[1]

Overall, however, natural pigments, despite their limitations, demonstrate immense potential as food colourings. They have tinctorial strengths that are comparable if not, superior to their synthetic counterparts[1].

In addition, they allow for more pastel, natural appearances that are believed to be of greater aesthetic value. Many of these pigments not only have no reported toxicity but are also suspected to be beneficial for optimal health[13,14].

1.3 COLOUR MEASUREMENT

Color perception is a “psychophysical” sense involving, the physics of light and objects as they interact, the physiology of the eye and brain and the psychology of the human mind. This is also known as the observer situation and is fundamental in the understanding of color and its measurement.



Observer ***Object/sample*** ***light source***

Fig 1.3 The observer situation

Earlier measurements of color by food industries were based entirely on this model (subjective visual inspection). This method is unfortunately limited by inconsistencies in viewing conditions, physiological limitations of the human eye including loss of color memory, color blindness and eye fatigue[15].

Objective methods of measurement were thus developed. These methods were based on instruments designed to emulate the mechanisms by which the human eye perceives color and on definitions laid down by the International Commission on Illumination (CIE). Such methods reproduce the observer situation but with the exclusion of ambiguities generated by differences in light source, the physical nature of the sample and in the psychology of the human mind[15,16,17].

An example of an instrument that accurately measures color in this manner is a spectrophotometer equipped with spectral sensors and software suitable for the

calculation of tristimulus values X, Y and Z and the conversion of these values to color spaces based on the CIELAB and/or CIE L*C*H models[5]

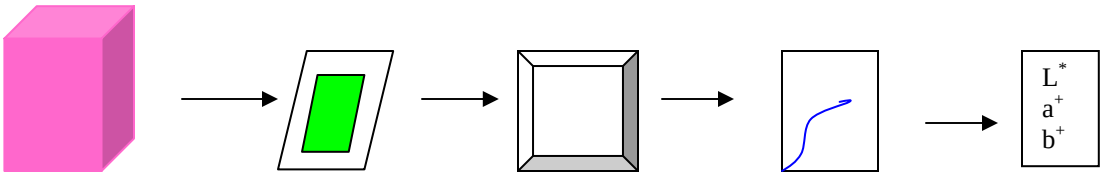


Fig 1.4 Schemetic representation of color measurement using a spectrophotometer equipped with a spectral sensor.

Tristimulus values arose from the trichromatic nature of the CIE system. It was postulated that any color could be represented as a mixture of 3 primary colors (or primaries). This postulate is reasonable because this is also the basis by which light induces the sensation of color in humans via the eyes. The 3 primaries are red, green and blue. These are designated as X, Y and Z respectively and the 3 primaries required to match a given color represent the tristimulus values of the color. Since various colors are associated with various wavelengths, it is also possible to indicate the amount of primaries at any particular wavelength. This is achieved using the *distribution coefficients x, y* and *z*, which are also known as red, green and blue factors respectively. The distribution coefficients for wavelengths contained in the visible spectrum are presented in Fig 3. Therefore, any color can be defined by its tristimulus values once their associated wavelength(s) has been identified. One problem with this system however, is

that the X, Y and Z values have no relationship to color as perceived, although a color has been completely defined. Color systems able to appropriately manipulate such data were thus developed[5].

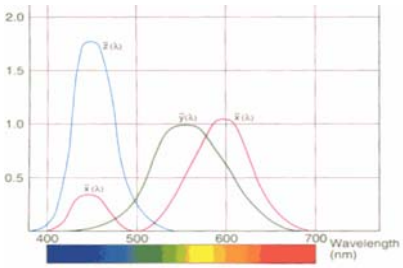


Fig 1.5 The values of the three distribution coefficients at various wavelengths in the visible spectrum.

The CIELAB and CIE L*C*h systems are the most extensively utilized for this purpose. The CIELAB system is also known as the Hunter L, a, b model. Here, the presence of an intermediate signal switching stage between the light receptors in the retina and the optic nerve, which transmits color signals to the brain, is assumed. In this switching mechanism, red responses are compared with green thus resulting in a red-to-green color dimension and yellow responses are compared with blue to give a yellow-to-blue dimension. These two color dimensions are represented by the symbols a and b. The third color dimension is lightness L[15].

Tristimulus values can be converted into L, a and b values using the following equations and vice versa[16]:

$$Y = 0.01L^{2} \qquad \textbf{[1]}$$

$$X = \frac{a + 1.75L}{5.645L + a - 3.012b} \qquad \textbf{[2]}$$

$$Z = \frac{1.786L}{5.645L + a - 3.012b} \qquad \textbf{[3]}$$

The L, a and b values can also be converted to a single color function (ΔE) using the relationship described on the following page:

$$\Delta E = (\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2 \qquad \textbf{[4]}$$

This difference is a measure of the difference between two colors only. It does not indicate the direction in which the colors differ on the Munsell color space.

The CIELAB system is often used together with a second color scale known as the CIE L*C*h or Munsell Color space (see Appendix1). This system is similar to the first but employs cylindrical co-ordinates instead of rectangular co-ordinates. It describes colors in terms of the parameters h (hue angle), c (chroma) and L (lightness) which are obtained from visual comparisons with color chips (Figure 1.6)[17]

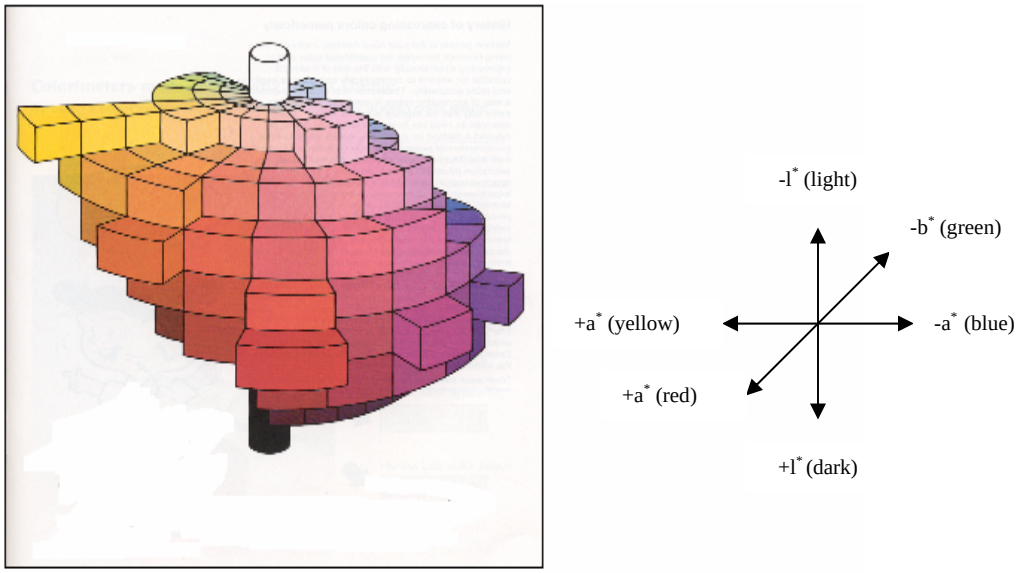


Fig 1.6 Munsell color chips, the basis of the Munsell color space. Defining axes shown on the bottom right-hand side of the diagram.

Hue is the name of a color such as red, green and so forth. Hues form what is known as the color wheel[18]. They are thus defined in terms of cylindrical co-ordinates. Hue angle is defined as starting at the +a* axis (0^o red), +b* axis (90^o yellow) and the -a* axis (270^o blue). Note that a hue angle of 360^o is equivalent to an angle of 0^o. This point has to be taken into consideration in the calculation of hue differences[17].

Chroma refers to the saturation of a color. It is neutral gray at the center (c*=0). Increasing chroma values corresponds to a transition from dull to vivid[18].

Lightness refers to whether the color being described is bright, mid-tone or dark. Lightness can be represented as a vertical scale, with white at the top, gray in the middle, and black at the bottom[17]. This parameter is common to both the CIELAB and CIE L*C*h color space.

Interestingly, it has been demonstrated that differences in hue are most readily noticed followed by differences in chroma and lastly, lightness. Hue angle and chroma can be converted into CIELAB values and subsequently to tristimulus values using the following equations:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \qquad \textbf{[5]}$$

$$h = \tan^{-1}\left(\frac{a^*}{b^*}\right) \qquad \textbf{[6]}$$

In addition to these features, spectrophotometers are built so that consistencies in viewing conditions are maintained (it should be noted that the Munsell system is a

visual color standard and may be used only under standard viewing conditions) and that differences in the physical nature of the sample are taken into account.

The physical nature of a sample is a point of importance in color measurement because it affects the way light interacts with the macromolecular make-up of the sample. Opaque samples reflect light. Therefore, powders for instance should be measured for reflected light because it is this scattered light, known as diffuse reflection, that is responsible for the color of a sample. Light absorbed by such samples, would never reach the eye for transmission to the brain, to generate the sensation of color. Similarly, the fact that transparent samples primarily transmit light while translucent samples both reflect and transmit light mean that only the appropriate light beams should be measured. Figure 1.5 illustrates the various ways light may interact with an object[15]:

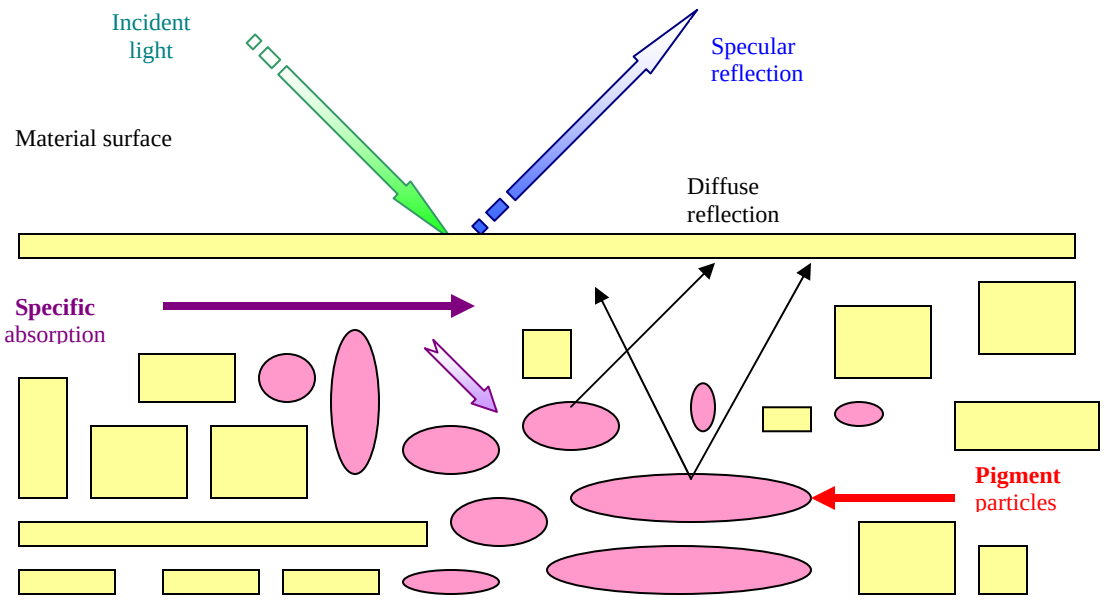


Fig 1.7. An illustration of the various ways in which light can interact with a given material

1.4 BETALAINS

In recent years, there is a tendency to limit the use of synthetic colours because of the safety concerns reflected in the new and tighter regulations existing in several countries[11]. This is particularly true for red colours, and therefore, it becomes necessary to seek alternative and additional sources that could be used by the food industry (Duxbury, 1990[11]). Betacyanins represent one such alternative.

Betacyanins constitute one of the two families of pigments that together, make up the class of red pigments known as betalains. Betalains are regarded as taxonomic markers for the centrosperma family. To date, more than 50 structures of naturally occurring Betalains have been elucidated. Betalains are frequently referred to by their common names that are usually assigned in agreement with their botanical genera. For instance, betanin, the most common betacyanin was first identified in the roots of *Beta vulgaris* whereas the betaxanthin, portulaxanthin was first isolated from the petals of *Portulaca grandiflora*[18].

Chemically, Betalains are immonium derivatives of betalamic acid.

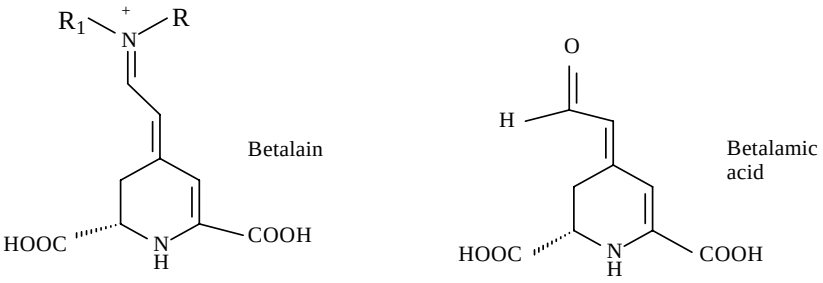


Fig 1.8 Representative structure of betalains (left) and betalamic acid (right)

Thus, the betalain chromophore is constituted by that of a protonated 1,2,4,7,7 pentasubstituted 1,7- diazaheptamethin molecular system [12,18].

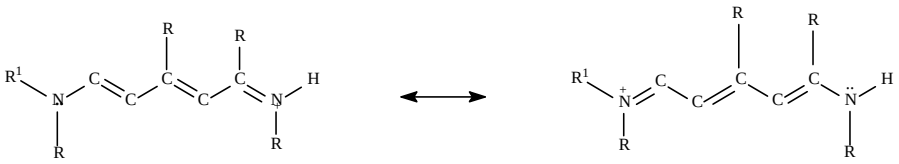


Fig 1.9 Canonical forms of a representative 1,7-diazaheptamethin molecular system.

Betaxanthins result when R^1 does not extend the conjugation. Hence, betaxanthins are yellow in appearance as the 1,2,4,7,7- pentasubstituted 1,7- diazaheptamethin chromophore exhibits an absorbance maximum in the 470-480 nm range.

Betacyanins result when R^1, in the form of a L-DOPA derivative (blue) extends the chromophore conjugation. The resulting chromophore displays an absorption maximum in the 530 to 540 nm range and hence, appear red. (Fig 1.10)

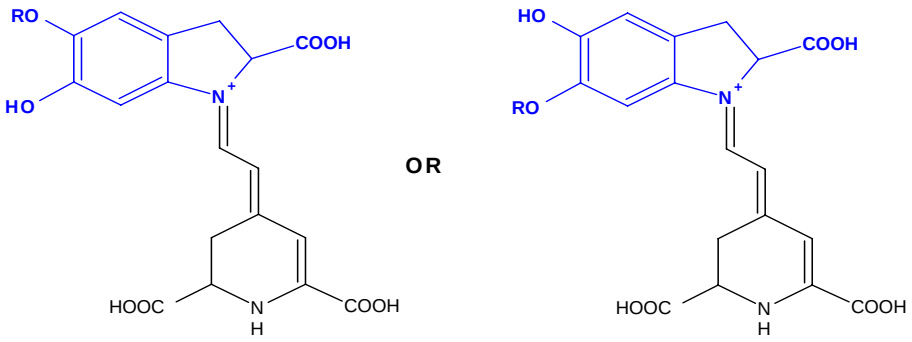


Fig 1.10 General structures of betacyanins.

The presence of a highly reactive, electrophilic immonium functional group in the Betalain structure suggests that they could be rather susceptible to nucleophilic attack. Indeed, this has been found to be the case as exemplified by the reaction cum destruction of the pigments by high water activity and pH ($[OH^-]$)[18]. The electrophilic moiety is also susceptible to reaction with atmospheric oxygen[18].

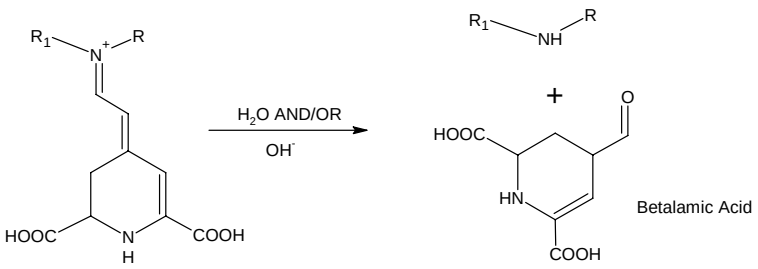


Fig 1.11(a) Hydrolysis of betalains into amines and betalamic

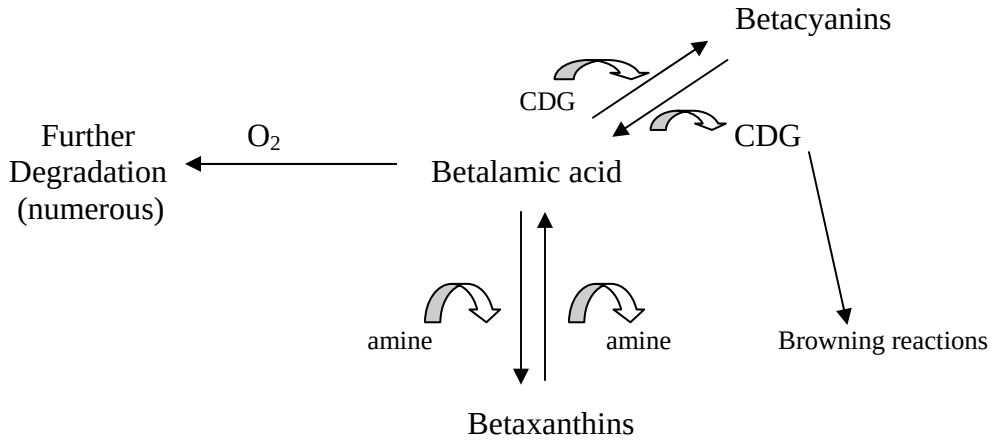


Fig 1.11(b) "Degradation Cascade" of betalains[18] (accelerated upon heating).

Nevertheless, the presence of the extensive Π-conjugation results in a significant moderation of the reactivity of the immonium functional group, particularly so in the case of the betacyanins as an outcome of the incorporation of an aromatic ring into the chromophore[14]. Hence, betacyanins are sufficiently stable to be able to function as food colorants in foods that experience minimal heat processing. This limitation is due to the susceptibility of betacyanins and of betalains, in general, to heat[1]. Examples of foods currently colored by betacyanins include fizzy drinks, wines, ice-cream, jellies, sweets and pastries.

Biosynthesis wise, betacyanins are derived from betanidin (2S, 15S) and isobetanidin (2S, 15R) by glycosidation of one of the phenol groups of the catechol moiety[12]. For instance, betanin, occurs as the 5-O-glucoside and gomphrenin-II, as a 6-O-glucoside. Generally, 5-O-glucosides are more common[12]. Betacyanins that are doubly-substituted with glycosyl moieties at both positions (in nature) have not been reported to date[12].

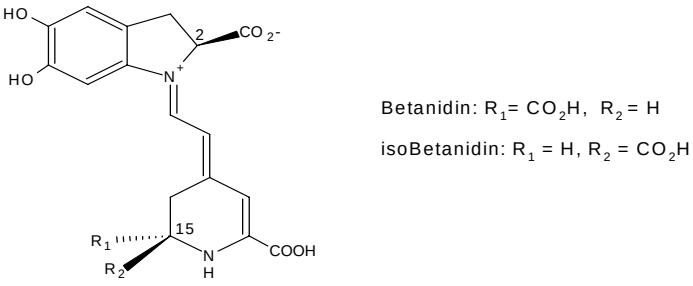


Fig 1.12 Molecular structure of betanin and betanidin.

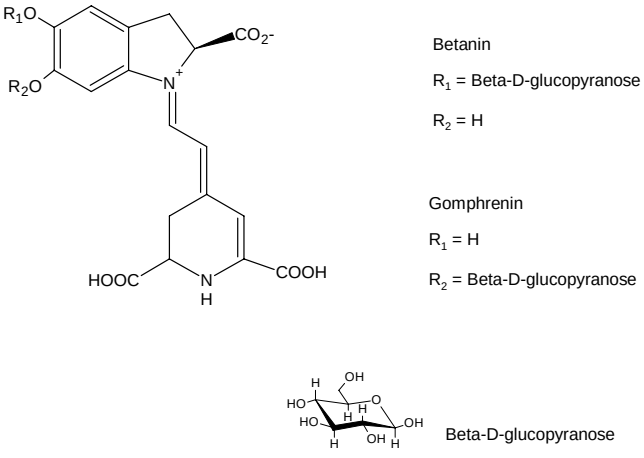


Fig 1.13 Molecular structure of betanin amd gomphrenin.

Betaxanthins, in contrast, are derived from Schiff base condensation of betalamic acids with amino acids, both proteingenic and non-proteingenic (Fig 1.8)[12]. They are

generally more susceptible to degradation than betacyanins. Interestingly, mixing betaxanthins with betacyanins diminishes the stability (color) of the latter[19].

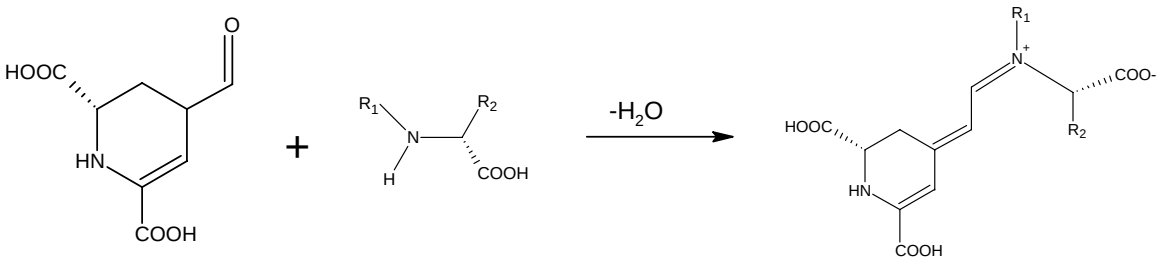


Fig 1.14 Formation of betaxanthins by Schiff base condensation.

2. LITERATURE REVIEW

2.1 DISCOVERY OF BETALAINS

It was evident to plant scientists, as far back as the 1930s, that there existed, in addition to the anthocyanins, a chemically distinct family of red pigments that was present in abundance in the roots of the red table beet (*Beta vulgaris*)[20]. They were commonly referred to as "nitrogeneous anthocyanins" to account for their anthocyanin-like colors and the fact that they contained nitrogen. The prevailing opinion of the era was that the "nitrogeneous anthocyanins" were of the following structure[20].

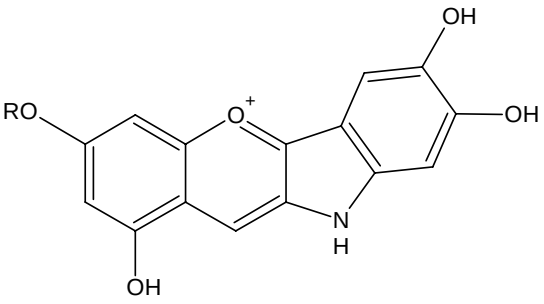


Fig 2.1 "Nitrogeneous anthocyanin".

This postulation had its basis in the knowledge that such compounds/pigments existed as glycosides, that they were positively charged (salt-like) and that cyclodopa (Fig 2.2) was produced from the degradation of pigment aglycones.

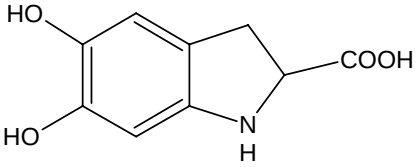


Fig 2.2 Molecular structure of CycloDopa.

It was the identification of 4-methylchelidamic acid (Fig 2.3) in addition to cyclodopa, as degradation products of the pigment aglycones by Wyler *et al* (1957)[20], that drew attention to the fact that the actual pigment structure could be rather different from the "nitrogeneous anthocyanin" picture. Wyler and Dreiding[20] concluded from these studies that the pigment aglycone contains three carboxylic acid, two aromatic rings and two aromatic hydroxyl groups.

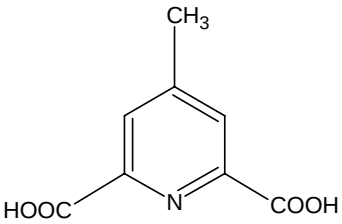


Fig 2.3 Molecular structure of 4-methyl Chelidamic acid.

It is interesting to note that unlike other natural products, direct characterization by NMR spectroscopy met with limited success due to the extremely poor solubility of the pigment and its aglycone in common organic solvents and the difficulty of obtaining a sufficiently dry compound. To date, the best (but nevertheless poor) spectrum has been obtained using a diluted solution of betanidin in *d*-trifluroacetic acid[22].

The mystery surrounding the actual molecular structure of betalains was finally solved in 1964, following a series of experiments by Mabry[23]. Mabry was able to obtain golden yellow crystals of a compound that was subsequently named pentamethylneobetanidin, by adding a few drops of an emulsion of the pigment aglycone (dispersed in aqueous methanol) to several liters of ethereal diazomethane (taking the necessary precautions) followed by overnight standing. The resulting

chloroform soluble crystals represented the first products from the pigment to contain all its carbon atoms and could be characterized by NMR Spectroscopy[22]. (Fig 2.4)

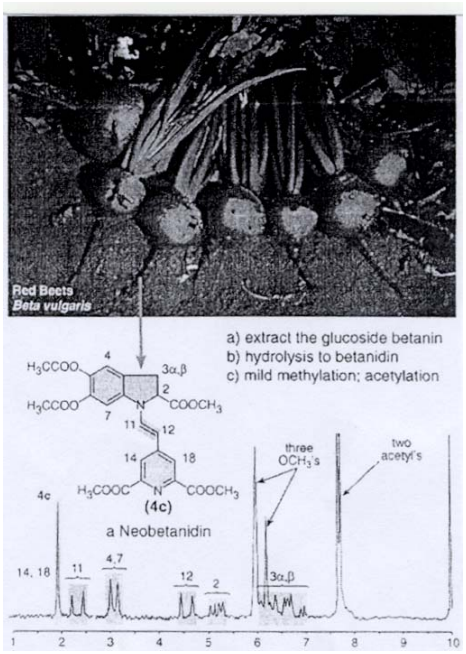


Fig 2.4 ^{1}H NMR spectra of pentamethylneobetanin[23]

Comparison of the resulting spectrum with those of cyclodopa, 4-methylchelidamic acid and the poorly resolved spectrum of the pigment aglycone, led Mabry and Dreiding[23] to conclude that the structure of the pigment was as shown in Fig 2.5.

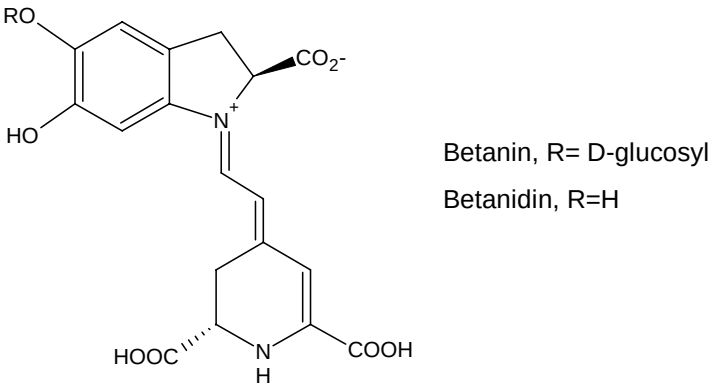


Fig 2.5 The structure of betanin and its aglycone, betanidin

The pigment was named betanin, and its aglycone, betanidin, by the duo. The stereochemistry of the glycosidic linkage (beta) was established using the appropriate glycosidase enzymes.

The chemistry underlying Mabry's experiments[23] is illustrated in Fig 2.6

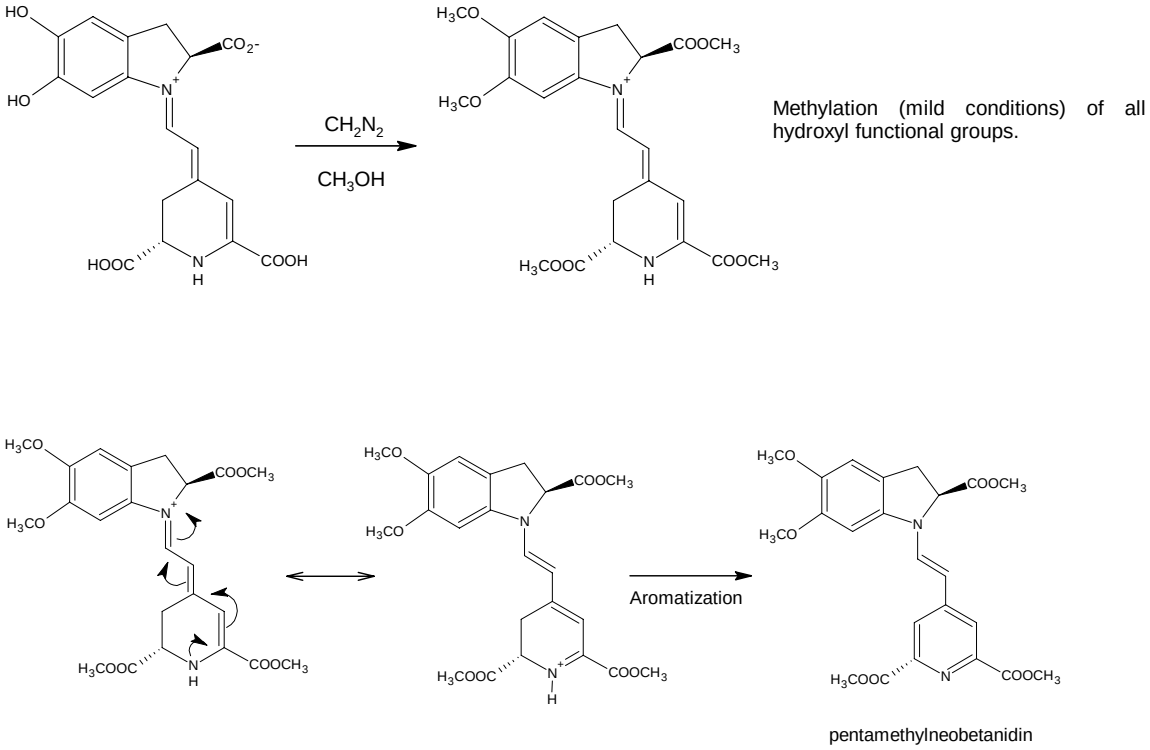


Fig 2.6. The chemistry behind Mabry's experiments in 1964.

In addition, the same workers, on the basis of feeding experiments postulated a biosynthetic pathway for betanin[12]. However, with the exception of the indicated glycosyltransferases, none of the enzymes in this Shikimate-based pathway have yet been isolated[14]. The proposed pathway is illustrated in Fig 2.7. Schiff base formation has been ascertained to be a non-enzymatic step in this pathway[24].

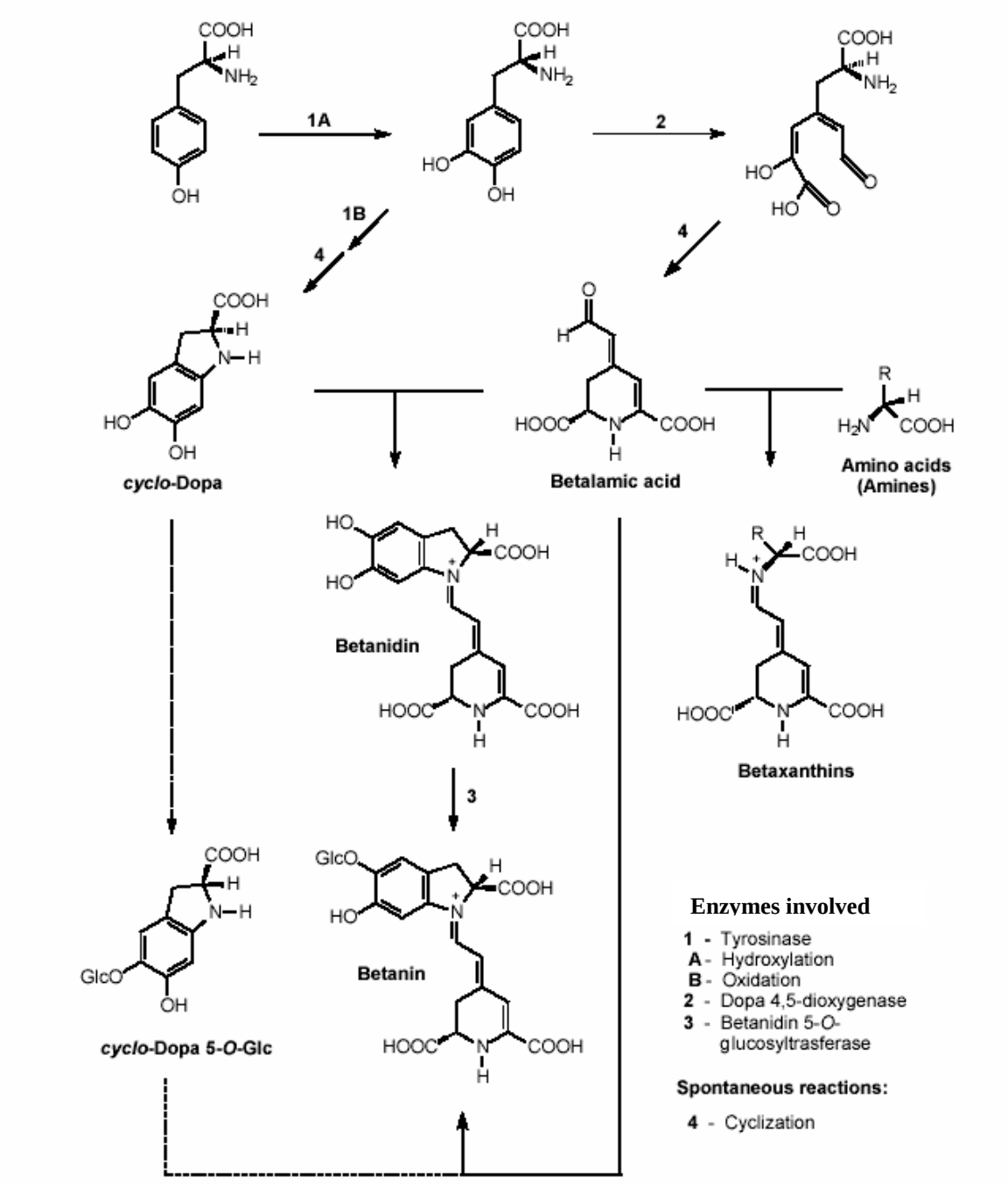


Fig 2.7 The proposed biosynthetic pathway for betalains.

2.2 DEVELOPMENTS FOLLOWING DISCOVERY

The molecular structure of betanin was solved by Mabry *et al* in 1964 using the techniques described in section 2.1. Henceforth, these workers proceeded to examine similar pigments from a variety of other sources using the same techniques/methodology. This was to culminate in the establishment of a library of analogous molecular structures for more than 50 betalain pigments nearly one decade later[12, 25]. It was soon recognized that betalains could be divided into two sub-families, the yellow betaxanthins and the red betacyanins. More importantly, the establishment of this library was instrumental in initiating a series of studies as regards the bioactivities, chemical ecology and food chemistry of the betalains. Standard analytical procedures for isolating, purifying and identifying betalain mixtures without the need for Mabry's dangerous but nonetheless, ground-breaking experiments were also developed.

2.2.1 BIOACTIVITY STUDIES

A variety of betalain-rich mixtures/isolates have been widely employed in traditional food products of some cultures and in the folkloric medicines of others[13,26]. For instance, jams made from entire fruits of *Hylocereus undatus* are used to color pastries and confectionaries in South America[26] while extracts have been used in folk medicine since ancient times mainly for cancer treatment as well as for the therapy of liver, spleen and skin diseases[13]. As such, it is not surprising that the earlier studies that were conducted in this area of work focused particularly on the toxicity/carcinogenicity and therapeutic aspects of the extracts[12].

Betalains appear to be non-toxic to humans given the fact that they are present in considerably high levels in many common foodstuffs such as beet root, prickly pears and Amaranthus seeds[27]. In fact, there is no known upper limit to the safe recommended daily intake[36]. Indeed, *in-vitro* assays involving five different strains of *Salmonella typhimurium* demonstrated the absence of mutagenic and carcinogenic activity for these bacteria in the betalains[28]. In addition, Schwartz *et al* (1983)[29] demonstrated that betalains do not initiate or promote hepatocarcinogenesis in diets containing up to 2g/Kg of betalains in a series of clinical trials but there is an occasional appearance of the pigment in the urine, an effect termed betaninuria or beeturia, a rare disorder whose mechanism and etiology remains shrouded in mystery.

Where therapeutic potential(s) are concerned, studies have demonstrated the immense potential of betalain extracts in cancer therapy/prevention. Studies have shown that betanin is capable of reducing lung carcinoma in rat models[14]. The ability of betalain extracts from beet root (90% betanin) to demonstrate significant anti-oxidant capacities (AOC) in ABTS assays over a wide pH range[14], coupled with their excellent oral bioavailability in human volunteers[30] demonstrates their immense potential as dietary anti-oxidants.

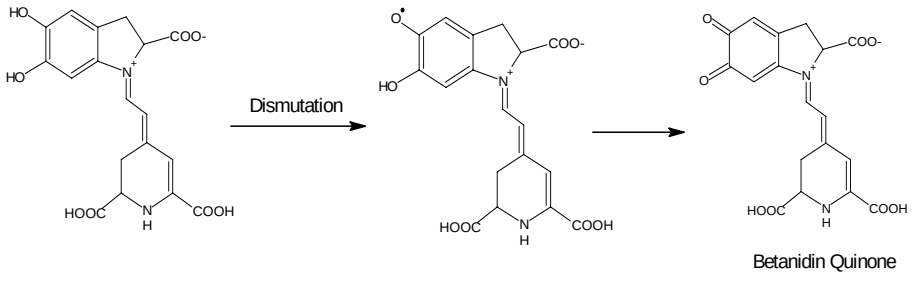


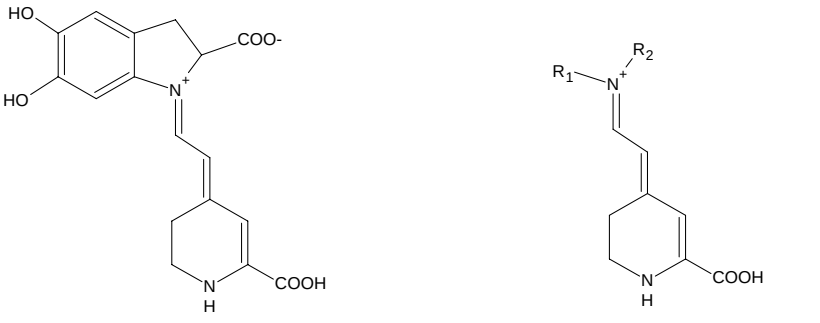
Fig 2.8 Quenching of radicals by betanidin[14]

To date, betalain extracts from beet root have been employed in pharmaceutical and nutraceutical preparations for use in cancer therapy/management[12].

2.2.2 DEVELOPMENTS IN THE FOOD CHEMISTRY OF BETALAINS

Betalain pigments, particularly the betacyanins, were already employed as food colorants prior to the elucidation of their molecular structures. This could be ascribed to the abundance of the pigment in the edible portions of a number of plants *e.g.* the roots of red table beets and in Amaranthus seeds[12]. Hence, studies pertaining to the chemistry of the pigments were commonly understood with regards to food systems. Specifically, the studies serve to provide a molecular perspective of the changes that occur under typical food processing conditions[18]. The elucidation of the structure of betanin and the subsequent establishment of a library, were invaluable to these efforts.

It was recognized that the chromophore of all betacyanins is constituted by betanidin, the aglycone of all betacyanins, while that for betaxanthins, the 1,2,4,7,7-pentasubstituted 1,7-diazaheptamethin system (below) provides the color base[12].



Betacyanin Chromophore

Betaxanthin Chromophore

Fig 2.9 Chromophore structures for betacyanins and betaxanthins

Generally, betalain extracts appear red or red-purple due to the abundance of betacyanins in them. Nilsson et al, 1970[31] designed a method based on UV-vis spectrophotometry that could quantify the two classes of pigments in a given extract without the need for prior separation. Nonetheless, it was also recognized that a "zone of weakness" in the form of a highly electrophilic immonium moiety exists within both chromophores. It was postulated that a nucleophilic attack on the immonium carbon would destroy the chromophore leading to a loss of color in the case of the betacyanins. Indeed, this was found to be the mechanism underlying the red to yellow transitions that is observed upon the excessive addition of alkali to a betacyanin solution[12]. (Fig 2.10)

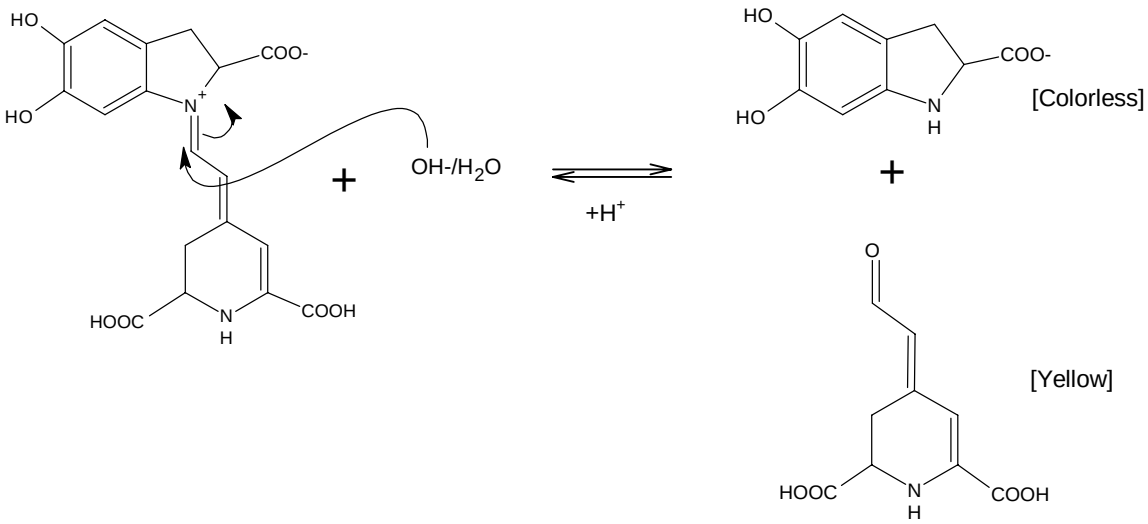


Fig 2.10 Hydrolysis of betanidin by alkali.

Betaxanthins undergo analogous degradation but the associated color changes are not easily observed with the naked eye. In addition, they are more susceptible to hydrolysis than betacyanins due to their less extensive conjugation[14]. Detailed studies

of the effect of pH on betalain stability using UV-vis spectrophotometry have been carried out by Von Elbe et al (1980)[18]. Such studies establish that the stability of the chromophore in the pH range of common foods (3 to 7) is preserved with instability arising only at pH>9.

As betacyanin degradation by high water activities, light, oxygen and heat are accompanied by similar color transitions, it is postulated but not demonstrated with the exception of high water activities, that such degradations are effected via similar mechanisms[18].

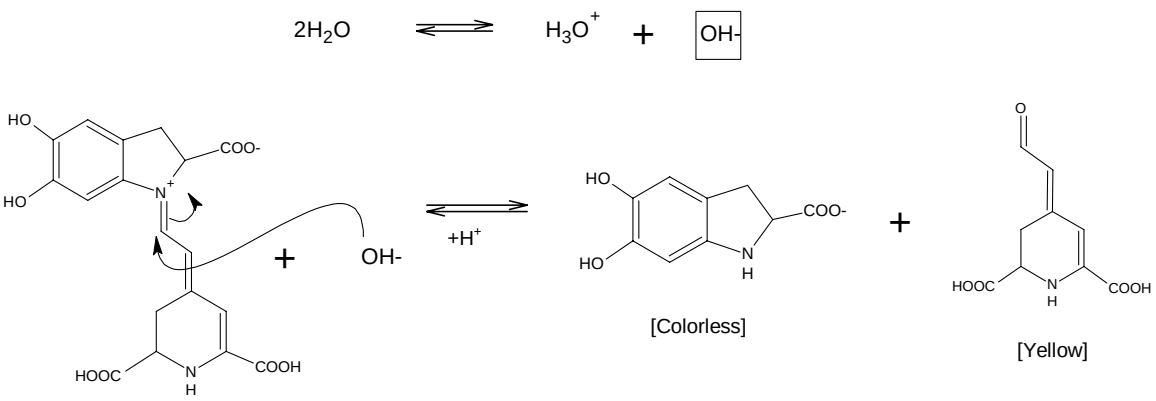


Fig 2.11 Degradation of betanidin in high A_w environments.

Degradations of betacyanins, in principle, is reversible via Schiff-base formation between the degradation products[18]. Indeed, recovery of the red coloration can be effected by rapid acidification and/or cooling following an increase in pH or temperature of a solution of betacyanin respectively[1]. (Fig 2.12)

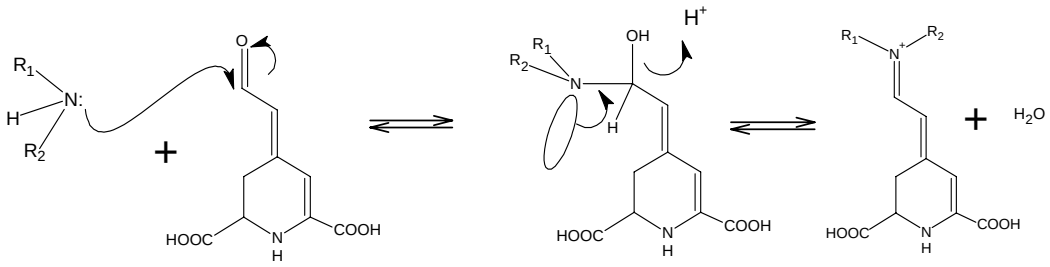


Fig 2.12 Pigment regeneration via Schiff base formation.

Nevertheless, the highly reactive nature of the aldehyde (in betalamic acid) and the catechol (in CDG) functional group, more often than not, results in a scenario in which the two degradation products are destroyed (via the degradation cascade mentioned in Chapter 1.4) faster than they are able to regenerate the pigment[18]. Thus, it may be surmised that the limitations of the betacyanins as food colorants is due to the reactive nature of the immonium functional group[10]. Hence, any attempt to widen the scope of application of the pigments should involve a supramolecular moderation of the immonium moiety's reactivity. Any such attempt, would be a challenging one given the fact that the potential preservative would have to be non-toxic and edible. To date and to the best of knowledge from a survey of relevant existing literature, appropriate preservatives have not been identified.

It is interesting to note that heating betacyanins (*in-vacuo*) under highly acidic conditions results in inversion at C15 and subsequently, decarboxylation[18]. This however, does not affect the color of the pigments since the chromophore is left intact.

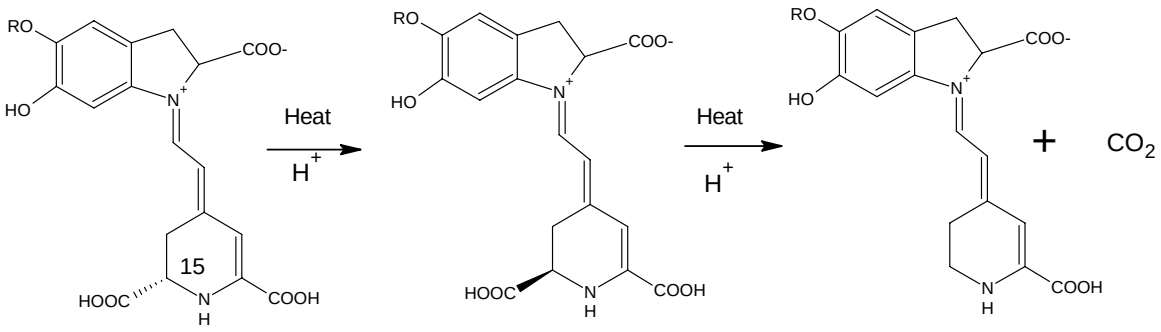


Fig 2.13 Decarboxylation of betacyanins.

2.2.4 DEVELOPMENT OF STANDARD ANALYTICAL PROCEDURES

The establishment of a library created much interest in the development of analytical procedures that are capable of separating complex mixtures of these pigments. It was envisioned that such methodologies would be invaluable to future phytochemical studies and product development applications as they are relatively safe and suited for use on a routine basis. Research in this area of study, has been facilitated by the work of organic chemists who were concurrently working on the chemistry of the pigments. Most notably, analytical chemists, armed with this plethora of information, were able to develop separation methods with the required efficiency whilst preserving the structural integrity (stability) of the pigments. In addition, an improved understanding of the chemo-enzymatic chemistry of the pigments culminated in the development of useful bioassays involving β-glucosidases[37]. Such assays are able to distinguish between acylated and non-acylated betacyanins[38]. (Fig 2.13)

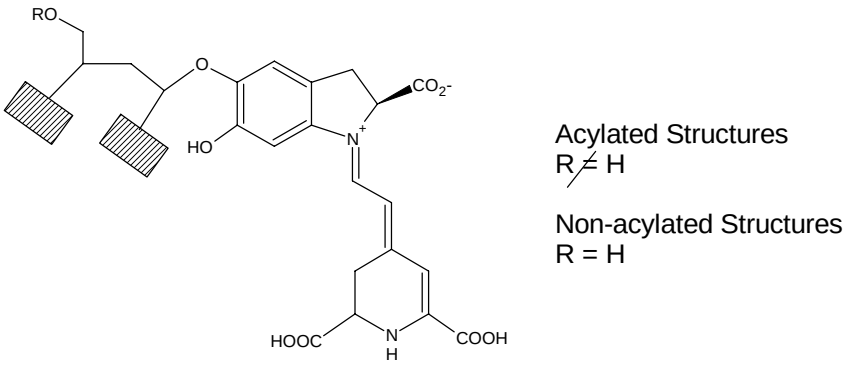


Fig 2.14 Representations of the structures of acylated and non-acylated betacyanins.

One of the earliest separation methods to be developed was based on paper electrophoresis[14]. Paper electrophoresis, however, was disadvantaged by its non-preparative nature and its relatively poor resolution. It was, for instance, incapable of separating betanin and its C15 epimer, isobetanin. In addition, satisfactory quantification was not possible. Paper electrophoresis was, however, capable of separating betaxanthins from betacyanins. This technique has been largely superceded by thin-layer-chromatography (TLC), which is a more convenient technique that is capable of greater resolution and preparative work when the appropriate high performance thin-layer-chromatography (HPTLC) and preparatory thin-layer-chromatography plates are employed. Nevertheless, the requirement for satisfactory quantification cannot be fulfilled by such methods.

The need for separation methods of adequate resolution and quantification was met with the advent of high performance liquid chromatography (HPLC) techniques. The earlier HPLC-based methods involved extraction(s) with aqueous alcohols and pre-concentration using ion-exchange column chromatography based on strongly acidic DOWEX 50-X2 cation-exchange resins[39]. This pre-concentration step was conducted at 10^oC to minimize pigment degradation and serves to separate

betacyanins from lesser quantities of betaxanthins and free sugars[39]. The sample was acidified to pH 3 prior to this step to optimize its interaction with the resins. The final analyses involving HPLC and a polyamide column (size-exclusion mechanism) were performed with this concentrate[39]. Such methods are based on the original preparative methods employed in the natural product-type studies of these pigments. These methods have evolved over the years, in part due to advances in the science of liquid chromatography and in response to the practical applications of the pigments, particularly as natural food colorants. To date, the analytical procedure(s) for studying pigment extracts may be summarized[14] as illustrated in Fig 2.15.

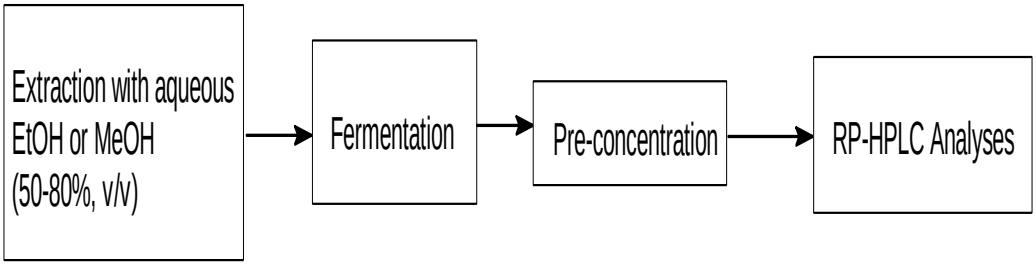


Fig 2.15 Typical procedure for the analysis of betalains.

Fermentation of the aqueous alcohol extract of betacyanins is commonly effected with microorganisms such as *Sacchromyces cerevisiae* and *Aspergillus niger*. It serves to increase pigment concentration in the aqueous alcohol extract by removing sugar impurities as ethanol and carbon dioxide. This procedure is perceived to be useful in improving the tinctorial strength of the extracts for application as natural food colorants[1]. Nevertheless, opinions regarding the actual usefulness of the procedure remain divided.

In contrast to the earlier methods, contemporary works[38,39,40,41,42,43] commonly involve cation-exchange and size exclusion column chromatography in sequence, for

pre-concentration purposes. The resulting concentrate would be subsequently analyzed by reverse phase (RP)-HPLC. Such methods, coupled with the appropriate detectors, afforded the necessary resolution for the complete separation of pigments including epimeric structures. Quantification could also be performed rather effectively with modern detectors such as photo diode arrays (PDAs) and hyphenated techniques such as involving mass spectrometers.

It is interesting to note that studies of betalain separation and quantification using capillary electrophoresis (CE) with PDA detectors have been conducted using beet root extracts as samples[13]. The results of the study are in close agreement with HPLC determinations. Nonetheless, the method of quantification differs slightly from those commonly employed in typical HPLC and CE works. Most notably, quantifications can be performed directly, using the molar absorption coefficient of betanin ($\varepsilon = 65000$), intensities of the relevant chromatogram peaks and Beer's law[13]. Nevertheless, there are discrepancies between such methods and the earlier mentioned UV-vis spectrophotometric methods [Nilsson *et al*, 1970][31]. These differences have been attributed to degradation products or interfering substances formed during processing[12].

2.3 AVENUES FOR FURTHER RESEARCH

Presently, research on betalains is largely fueled by the interest and demand for natural food colorants. Betalains, in the form of beet juice concentrate, are well suited for such purposes due to their demonstrated stability over the pH range (3 to 7) of common foods and more importantly, their non-toxicity[14]. Hence, beet juice concentrates have been employed for many years as natural red, food colorants.

Nonetheless, shortfalls exist in the form of short shelf-life and susceptibility of the betalains[44] to elevated temperatures, light and oxygen. As such, beet juice concentrates have had their scope of application limited to food products such as ice-cream, fizzy drinks, sweets and confectionaries[1]. Much effort as such, has been focused on pigment preservation.

One of the earliest efforts was undertaken in the light of the knowledge that the proportion of betaxanthins and metal ions in pigment extracts could be inversely related to the hue stability of the extract[5, 44]. Macro-scale purification methods for the removal of such impurities have been developed. Nevertheless, the benefit(s) of such purification on the hue stability of beet juice concentrates is debatable. This suggests that the inherent instability of hue in beet juice concentrates could be due to other factors, most notably, the presence of degradative enzymes in pigment extracts[15]. This has culminated in the development of extraction methods that serve to denature enzymes, for instance, using alcohols as extracting solvents as well as the investigation of alternative pigment (betalain) sources.

Members of the cactaceae constitute promising alternatives[38,39,40,41,42,44,45]. Pigment extracts from such sources are devoid of betaxanthins and are known to be of better stability than beet juice concentrates. Hence, the need for betaxanthin-removing purification procedures is obviated for such extracts. HPLC-PDA and LC-MS analyses have established that such extracts, like those of beet root, are largely constituted by betanin[15]. To date, the fruits of the *Opuntia ficus*, a cactus, have been approved for use as a food colorant by the FDA[14]. However, other members of the family would seem to merit further investigation.

Reports on pigment preservation using approaches based on molecular strategies have been scarce. This is largely due to the highly reactive (electrophilic) nature of the immonium moiety in betalains which renders any such attempt highly challenging as mentioned earlier. Hence, there is much room for impacting research in this area of work.

Assaying natural products for biological activity and/or potential biological applications has always been an ongoing endeavor in many research groups. This is also true for the betalains. Indeed, the largely planar, positively charged molecular structure of betanidin suggests that it might be capable of interacting with DNA[42] with the potential for use as a molecular scaffold for assembling general medicinal and possibly, specific anti-cancer drugs. Thus far however, work of this nature has not been reported possibly due to the absence of synthetic strategies for this class of pigments. Nevertheless, there have been attempts to employ betalains as nutraceuticals[12]. Here, betalains are employed as a vehicle to deliver/reinforce the levels of specific amino acids in diets. This is done via carefully controlled, alkaline (ammonia) hydrolysis of betanin, isolation of betalamic acid followed by its recombination with selected amino acids via Schiff base condensation[12]. The resulting betalains (betaxanthins) would then effect dual purposes as food colorants and as nutraceuticals (Fig 2.15).

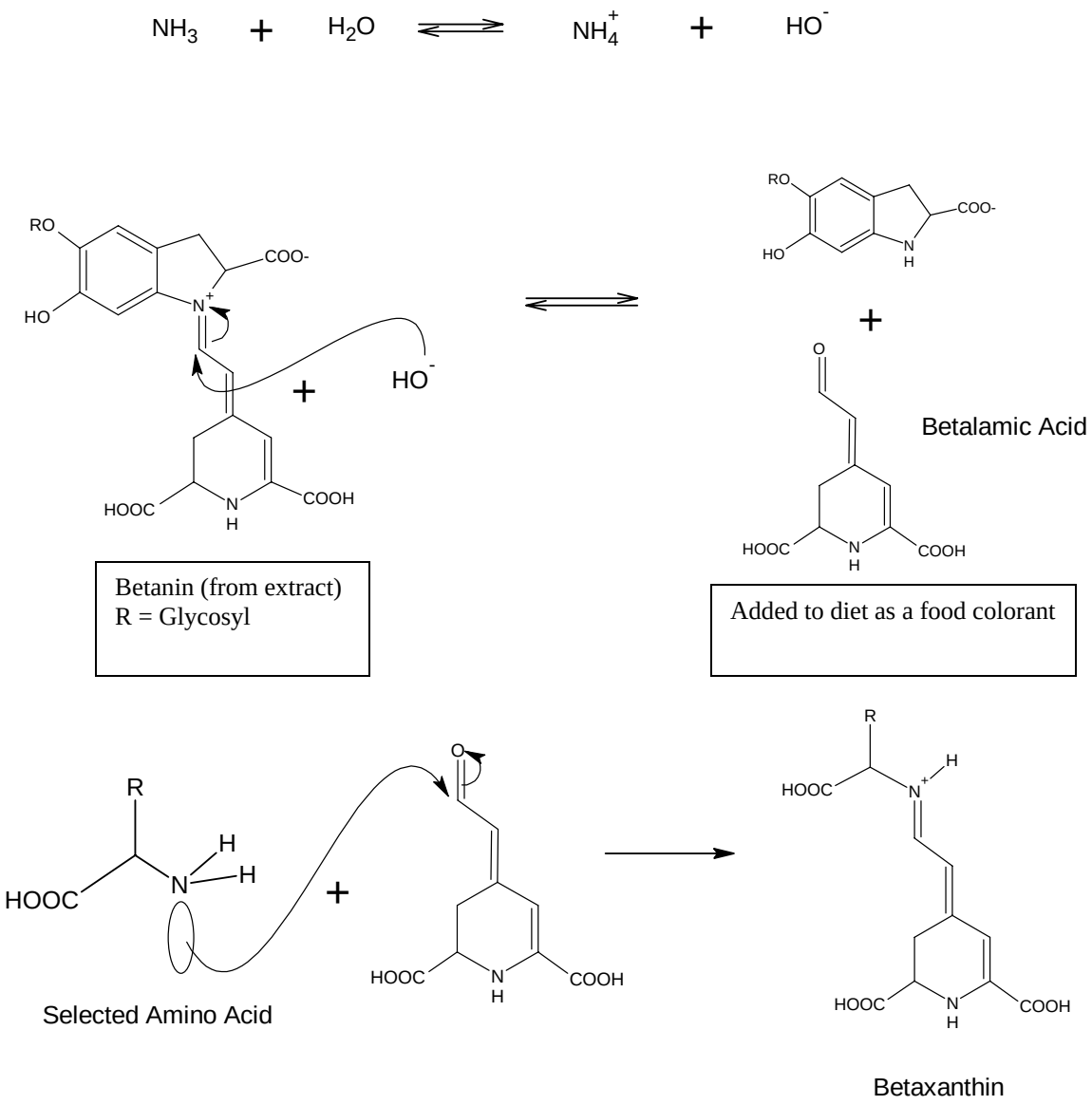


Fig 2.16 Preparing betaxanthins for the delivery of specific amino acids

In summary, in addition to the traditionally researched areas of pigment preservation, there is much scope for studies involving biological activities, for instance, in the development of nutraceuticals and drugs where betalains research is concerned.

3. AIMS AND OBJECTIVES

3. AIMS AND OBJECTIVES

The principle objective of this work is to investigate the feasibility of employing *Hylocereus undatus* (Dragon fruit or Pitaya) harvested in Vietnam as a source of betacyanin pigments for use in food products. Such use, utilizing the waste remains of the plant would suggest an economic advantage. *Hylocereus undatus* is an epiphytic cactus. The cacti fruit, commonly referred to as the *Pitaya*, Dragon fruit or Strawberry pear[26] is harvested as a food crop, in quantities as large as 100,000 tons/year, in Vietnam[47]. The non-spiny fruit is oblong-oval, up to 10 cm long, 6.25 cm thick, coated with the bright-red, fleshy or yellow, ovate bases of scales. Within is the white, juicy, sweet pulp containing innumerable tiny black, partly hollow seeds[26].

The fruit peel is usually not consumed (it is not unusual for inner layers of the peel to be ingested unintentionally when the fruit flesh is being scrapped off for consumption) and is commonly discarded although there are unconfirmed reports[26] of syrups made of the whole fruit being used to color candies and pastries. This means that a significant proportion of waste is being created. This waste, comprising tons of pinkish red peels could ironically be a rich source of natural colorings (betacyanins). To date, the vast majority of reported works on betacyanins extracted from members of the *Hylocereus* genera has been focused upon qualitative analysis/identification of specific betacyanins in the extract. There are no known reports on the performance of the extracts as natural food colorants.

The chemistry of betacyanins will be reviewed and discussed leading to the development of extraction methods of betacyanins from Dragon fruit peel with a

possible industrial/application bias will be undertaken in this project. The stability of the hues of pigment extracts during aging, storage, exposure to light and elevated temperatures for varying durations will also be examined and compared with betacyanins derived from beet juice powder, the only known commercial betacyanin food colorant to date.

4. MATERIALS AND METHODS

4.1 REAGENTS AND SOLVENTS

4.1.1 SOLVENTS

Ethanol(99.9%, v/v) was purchased from Tedia Company Inc (USA). Chloroform (99.8%, w/v) was obtained from J.T Baker (USA). All aqueous solvents were generated using deionized (DI) water.

4.1.2 pH ADJUSTMENTS

Concentrated phosphoric acid was purchased from BP Chemicals (Singapore). Aqueous ammonia was obtained from J.T Baker (USA).

4.1.3 THIN LAYER CHROMATOGRAPHY (TLC) PLATES

RP-TLC plates in the form of RP-18 F_{254s} were obtained from Merck (Germany).

4.14 COMMERCIAL BEET JUICE POWDER

This was manufactured by W. Schoenenberger Pflanzensaftwerk, D-71106 Magstadt- Germany. Suitable quantities were dissolved into DI water to generate standards for comparison purposes in the following analyses.

4.2 INSTRUMENTATION

4.2.1 MEASUREMENT OF HUE (COLOR SHADE)

A Minolta CM 3500d spectrophotometer (Japan) equipped with a spectral sensor (Dual 18-element silicon photodiode array with wedge-shaped continuous interference filter) for measurement of tristimulus values was used to describe the

color appearance of the samples. These values were electronically converted into CIE L*a*b* and CIE L*C*h notations. The hue angle/value was subsequently obtained.

4.2.2 ROTATORY EVAPORATION

Concentration of extracts or solvent removal was performed using a Buchi RE120 rotatory evaporator (Switzerland). The vacuum pump used was a Buchi B-169 vacuum system. The condenser was maintained at temperature of 14 ^{o}C by a Thermomix cooling unit from B. Braun Biotech International (Germany). The water bath unit of the rotatory evaporator was maintained at 35 ^{o}C by a built in thermostat.

4.2.3 FREEZE-DRYING

A Virtis Advantage freeze dryer (Switzerland) powered by an Edwards AV 3 (England) vacuum pump was employed. A cold trap based on an acetone-dry ice mixture was incorporated as a protective measure for the pump.

4.2.4 pH MEASUREMENTS

The pH probe employed in this study was a Thermo Orion model 410 (Switzerland). This was calibrated using Metrohm pH 7.00 and pH 4.00 buffers purchased from Merck (Germany).

4.2.5 CENTRIFUGATION

Centrifugation under isothermal conditions was performed using an Eppendorf^{TM} 5804 centrifuge programmed at 4^{o}C, 4500g and 30minutes.

4.3 SAMPLE PREPARATION

Dragon fruits, imported from Vietnam, were purchased from a local wholesaler's market. Each extraction process would involve six fruits. Fruits were washed and cut into smaller pieces following which, the whitish flesh and tiny black seeds were meticulously scrapped off with a knife. Accidental removal of other parts of the fruits was carefully avoided. The peels were homogenized/macerated using a Braun MR430HC/AC Multiquick Deluxe hand kitchen blender (Germany).

4.4 EXTRACTION METHODS

4.4.1 EXTRACTION PROTCOL I

Macerated fruit peels were extracted by overnight stirring in DI water at room temperature (28 ^{o}C). The pinkish supernatant was isolated by vacuum filtration using glass wool in place of filter paper. The gelatinous filtrate was stored in the dark at 2 ^{o}C until required for analysis.

4.4.2 EXTRACTION PROTOCOL II

Macerated fruit peels were extracted by overnight stirring in 70% (v/v) ethanol at room temperature (28 ^{o}C). The reddish pink supernatant was isolated by vacuum filtration using Whatman^{TM} No.4 filter paper. This was concentrated *in-vacuo* using a rotatory evaporator with a bath temperature of 30 ^{o}C. Betacyanins were precipitated from the concentrate by absolute ethanol addition (3: 1, v/v) followed by overnight refrigeration at 4 ^{o}C. The precipitate (BetX) was isolated by centrifugation at 4^{o}C and washed with ice-cold absolute ethanol. This precipitate was then freeze-dried and

dissolved in DI water to generate 0.5% (w/v) and 0.01% (w/v) solutions and stored in the dark at 2^{o}C until required subsequently for shelf-life and temperature studies respectively.

4.4.3 EXTRACTION PROTOCOL III

Macerated fruit peels were extracted by overnight stirring in 70% (v/v) ethanol at room temperature (28 ^{o}C). The reddish pink supernatant was isolated by vacuum filtration using Whatman^{TM} No.4 filter paper. This was concentrated *in-vacuo* using a rotatory evaporator with a bath temperature of 30 ^{o}C. Clean-up of the concentrate was effected by multiple (six) solvent extractions using chloroform. Betacyanins were precipitated from the partially purified concentrate by absolute ethanol addition (3:1, v/v) followed by overnight refrigeration at 4 ^{o}C. The precipitate (BetX) was isolated by centrifugation at 4 ^{o}C and washed with ice-cold absolute ethanol. This precipitate was then freeze-dried and dissolved in DI water to generate solutions that were of concentrations 0.5% (w/v) and 0.01% (w/v) and stored in the dark at 2 ^{o}C until required subsequently for shelf-life and temperature study purposes respectively.

4.5 ANALYSIS

All measurements were conducted in triplicates and mean values calculated.

4.5.1 VISUAL CORRELATION

A yardstick for visual correlation purposes was generated by doping 0.01% (w/v) BetX with aqueous ammonia and/or phosphoric acid to generate solutions with

integer pH values ranging from 3 to 13. The solutions were photographed and the hue value of the solutions at each pH was recorded. A plot of percentage change in hue (%dH) against pH was established.

4.5.2 TEMPERATURE STUDIES (Appendix)

Sample solutions containing 0.01% (w/v) of BetX from extraction protocols I and III and solutions of commercial beet juice powder (0.01%, w/v) were adjusted to generate sample solutions with integer pH values ranging from 3 to 7 using phosphoric acid and/or aqueous ammonia. Each solution was exposed to elevated temperatures of 50^{o}C, 70^{o}C and 90^{o}C for up to 30 minutes. Hue values were recorded at regular intervals of 5 minutes from 0 to 30 minutes inclusive. Percentage changes in hue values (%dH) were calculated and a plot of %H against time established.

4.5.3 SHELF LIFE STUDIES

Sample solutions containing 0.01% (w/v) of BetX from extraction protocols II and III and solutions of commercial beet juice powder (0.5%, w/v) were prepared. Sample solutions were stored under (i) darkness at room temperature (28^{o}C), (ii) exposed to the surroundings i.e. to 40W fluorescent light tubes and at room temperature (28^{o}C) and (iii) darkness at 2^{o}C. The sample solutions were inspected on a daily basis. The number of days for which the sample solutions could retain their reddish pink appearances was taken as the apparent shelf life of the respective solutions.

5. RESULTS AND DISCUSSION

5.1 PRELIMINARY STUDIES

The conduct of appropriate feasibility studies, as regards the use of betacyanin extracts from *H.undatus* as food colorants, constituted initial efforts in this study. The focus of such studies, revolved around the stability of such pigment extracts and hence, their associated hue(s) following exposure to commonly encountered food processing and storage conditions[6]. The use of a spectrophotometer as the principle instrument for analysis was decided upon[17].

In these studies, extraction was performed using aqueous methanol (70%, v/v). The aqueous extract was concentrated *in-vacuo* with subsequent clean-up effected via solvent extraction(s) using *n*-hexane (85%, v/v). Betacyanins were precipitated from this partially purified extract using absolute ethanol at 4^{o}C as described in Chapter 4. Sample solutions for analyses were prepared accordingly. Parameters of interest included hue stability during long-term storage under commonly encountered storage temperatures, following exposure to elevated temperatures and the effect of light. The results of these preliminary studies[48] will be reviewed in this section (5.1).

5.1.1 HUE STABILITY DURING STORAGE

Changes in hue, of solutions of betacyanin extracts derived from dragon fruit peel, were found to be marginal for as long as a month, when these were stored at room temperature (28 ^{o}C). The percentage change in hue for samples stored in the dark and when left exposed to room lighting was –3.66% and –2.40% respectively. Samples stored in the dark at 0^{o}C for a similar time period exhibited a percentage change in

hue of –1.29%. Visually, all three samples retained their original red-pink hue after a month of storage. Thus, hue stability appears to be sufficient for long term storage of such aqueous pigment extracts.

5.1.2 HUE STABILITY IN THE pH ENVIRONMENT OF COMMON FOOD SYSTEMS

The pH environment of common food systems has been determined to be in the range of 3 to 7 as mentioned in chapter 2. For the aqueous extracts (of BetX) to be able to function as natural food colorants, appreciable stability of hue in this range of pH should exist. As indicated in fig 5.1, changes in hue were marginal, varying between ±2.50%. Therefore, the aqueous extracts do possess adequate hue stability in the above mentioned pH range, and hence, in the pH environment of most food systems.

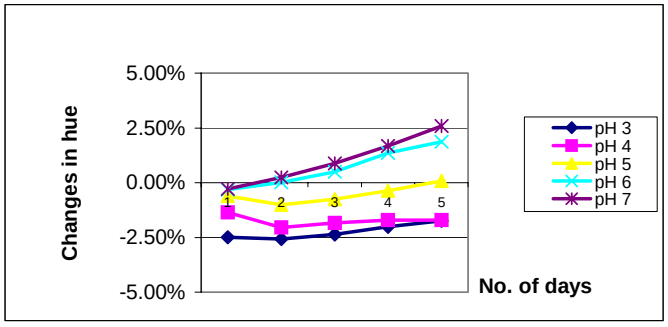


Fig 5.1Changes in hue as a function of changing pH of 0.01% (w/v) BetX.

5.1.3 METAL ION STUDIES

It has been reported that the presence of various metal ion species in a given food system could lead to disturbance of the system via the ability of metal ions to induce precipitation (for aqueous systems) and via their ability to catalyze various degradative mechanisms.

In this investigation, the effects of Al^{3+}, Cu^{2+}, Fe^{2+} and Sn^{2+} on the stability of the color shade of 0.01% (w/v) BetX was examined. These metal ions were selected for their possible occurrences in biological (food) environments in the case of Cu^{2+} and Fe^{2+} or in food containers in the case of Al^{3+} and Sn^{2+}. The quantities of metal ions used were in accordance with levels permitted by local regulations (Refer to table 2). A 0.01% (w/v) BetX with no added metal ions was employed for blank analysis.

Metal ion species	Quantity permitted under Singapore food regulations	Quantity used in experimental work
Al^{3+}	60ppm	50ppm
Cu^{2+}	30ppm	20ppm
Fe^{2+}	30ppm	20ppm
Sn^{2+}	250ppm	200ppm

Table 2 Selected metal ions employed in the study

Hue was found to be susceptible to additions of Cu^{2+},Fe^{2+} and Al^{3+} over a five-day period. Hue was however, stable to additions of Sn^{2+} over the same time period (Refer to figure 5.2)

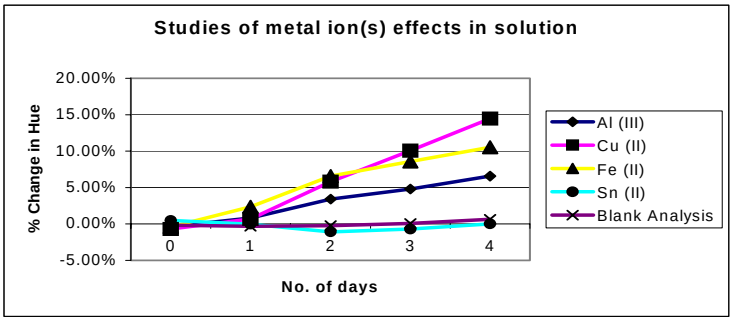


Fig 5.2 Changes in hue of 0.01% (w/v) BetX following exposure to selected metal ion species.

Hue stability of similar additions to gelatin solutions that were eventually cooled to form jellies was however, much higher. Hue changes were much lower over the same time period (Fig 5.3). This could be due to the constrained environment

presented by the gelatin network since chemical reactivity between the various additives would be hindered. Since similar environments are likely to be present in most foods (not necessarily due to gelatin but to other macromolecules such as carbohydrates, proteins etc), hue stability is likely to be preserved in most applications.

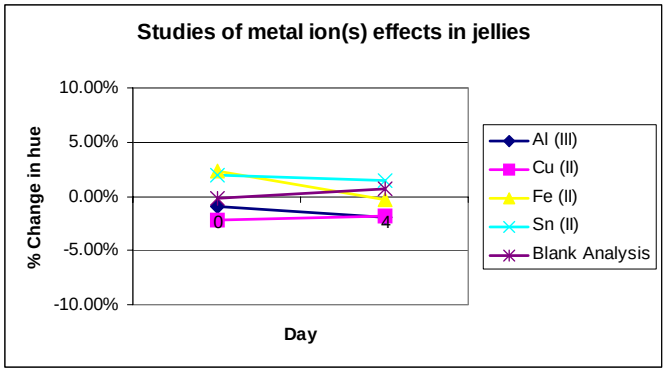


Fig 5.3. Changes in hue of jellies colored using 0.01% BetX spiked with selected metal ion species.

5.1.4 TEMPERATURE STUDIES

In this study, aqueous solutions of BetX (0.01%, w/v) were exposed to selected temperatures for various durations. Hue stability to elevated temperatures was generally, poor. Loss of hue was particularly significant when the exposure time was prolonged. Thus, exposure to 100 ^{o}C (45mins) results in a transition to yellow hue, exposure to 60 ^{o}C (6mins) results in an orange hue while exposure to 45 ^{o}C (12 hours) results in a yellow orange hue. A better retention of the original hue was achieved when the exposure time was kept short. This was supported by the appearance of the sample (fig 5.4a) exposed to 80 ^{o}C (8mins). Recovery of hue was not observed despite the fact that cooling (refrigeration at 4 ^{o}C) was effected immediately after the heating process. Stabilizers in the form of ascorbic acid (0.01%, w/v) improved hue

stability marginally. Quantitative improvement (fig 5.5) was achieved although it was definitely inadequate for the purpose of hue conservation.

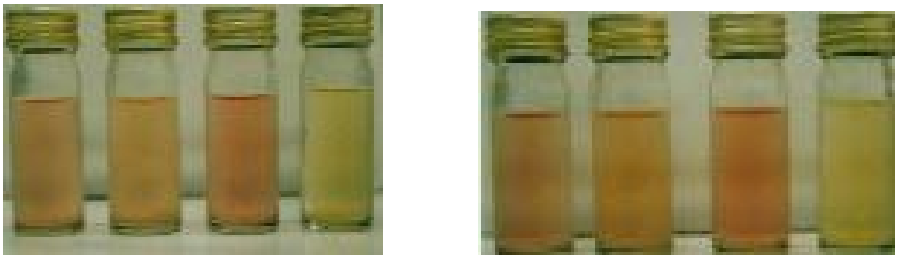


Fig 5.4(a) After exposure appearances of test samples subjected to (left to right) 45^{o}C (12 hours), 60^{o}C (6 hours), 80^{0}C (8 mins) and 100^{o}C (45 mins) respectively. Samples in the picture on the left do not contain ascorbic acid whereas those in the right picture are added with 0.1% (w/v) of ascorbic acid

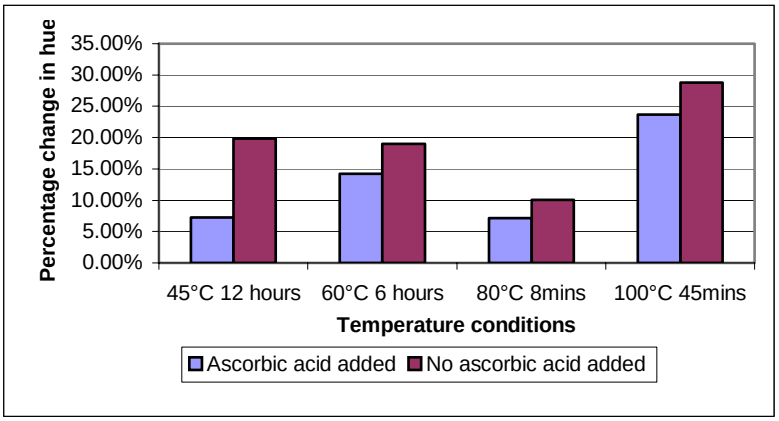


Fig 5.5 Graphical illustration of the effects of ascorbic acid on hue conservation following the exposure of BetX solutions to elevated temperatures.

In addition, the preliminary study identified the crucial finding that the hue stability exhibited by BetX solutions stored at room temperature, was much higher than that of beet juice concentrate. Encouraged by these findings, an attempt was made to examine the pigments in greater detail. There were particular interests in the formulation of pigment extraction strategies with an industrial bias and in probing the limits of hue stability following exposure to elevated temperatures.

5.2 EXTRACTION

In view of the fact that product development constitutes the long-term objective of this study, the preparation of pigment extracts, i.e. the extraction process is of optimum importance. In particular, any proposed protocol should involve only chemicals/materials and procedures that are compliant with local (Singapore) food regulations[27], those of the United States Food and Drug Administration (FDA)[50] and the European Union (EU)[50].

In addition, two fundamental aspects were kept in mind in the formulation of a suitable extraction strategy, namely; can the analyte (betacyanins) of interest be isolated? How (if any) does the extraction methodology affect the performance of the extracts as natural food colorants?

As betacyanins are brightly colored, it is relatively simple to keep track of the first aspect. For this purpose, qualitative analyses in the form of RP-TLC[48] were extensively employed in addition to visual inspections. 80% ethanol (v/v) was employed as the mobile phase with commercial beet juice concentrates as a reference. A co-spot consisting of pigment extract and commercial beet juice concentrate was also employed. (Fig 5.6)

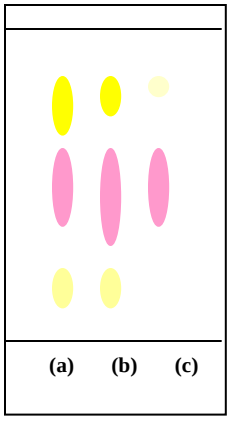


Fig 5.6. Typical RP-Thin Layer Chromatogram for (left to right); (a) commercial beet juice concentrate (b) co-spot consisting of a spot of (a) and (c), and (c) pigment extracts from Dragon fruit peel. (R_f for pink [betacyanin] spot *c.a.* 0.25 to 0.33)

In addition, the extracts were ascertained to consist largely of betacyanins using simple chemical tests[12]. Specifically, the red-pink extracts turned yellow following the addition of OH^- with the original red-pink color restored upon the addition of an acid. It was verified that 70% (v/v) ethanol and DI water could successfully isolate betacyanins from the fruit peel although with the latter; the intensity of the extract was observably lighter with the solvent extract appearing pinkish instead of the usual red-purple coloration.

The choice of 70% (v/v) ethanol and separately, DI water as extracting solvents was guided largely by the fact that the eventual application of the pigment extract will be in foods. Both solvents were able to isolate the desired analytes under the indicated experimental conditions. In addition, both are of relatively low toxicity. As such, the inevitable occurrence of residual levels of solvents, in pigment extracts, would be less likely to be a hazard during their application as food colorants, as opposed to when a toxic solvent such as methanol is being employed. The auto-dissociation of water and the subsequent generation of nucleophilic hydroxyl species when water is employed as the extracting solvent should not be a cause for concern, since extraction was performed at low temperatures of *c.a.* 10^oC as reported in earlier works. With regards to costs, ethanol is more costly than DI water as an extraction medium. However, it should be kept in mind that subsequent drying processes for water extracts would be energetically greater and hence, more costly[55]. As such, the selection of an extracting solvent would be more appropriately based on performance factors.

Concentration of the solvent extract by rotatory evaporation was performed at a bath temperature of 35^oC. Deliberate avoidance throughout the study of temperatures

greater than 40^{o}C was undertaken to minimize possible thermal degradation of the extracted pigments[51]. A pink residue (BetX) in a clear yellow supernatant was subsequently produced upon the addition of absolute ethanol, in excess to the concentrate followed by overnight refrigeration at 4^{o}C. The pink residue was isolated by centrifugation and rinsed with ice-cold absolute ethanol. This was then freeze dried for 12 hours. However, an alternative drying technique, spray drying using cyclodextrin carriers, is more popular industrially due to energy costs[1]. This technique was not utilized in this thesis research however, as appropriate instrumentation was not available during the entire course of study.

Protocol III differed slightly from protocol II in that in the former, the initial solvent extract was partially purified by multiple solvent extractions with chloroform prior to precipitation with absolute ethanol. The purpose of this added modification was to utilize the excellent properties of chloroform as a solvent to generate pigment extracts with a higher level of purity for the purpose of comparing performances.

In view of RP-TLC results and the physical as well as solution properties of betacyanins in general, it might be assumed that the pink residue obtained at the final stages of the extraction protocol consists pre-dominantly of betacyanins. Solutions containing 0.5% (w/v) and 0.01% (w/v) BetX were prepared for shelf life and temperature studies respectively. The choice of employing solutions with concentrations of 0.5% (w/v) and 0.01% (w/v) was based largely on common practices[1,44] in which pigment solutions for storage are prepared by dissolving dried pigment extracts in water to yield solutions containing 0.5-1.0% of solids. These are commonly diluted by approximately 10 times before application in food systems.

5.3 ANALYTICAL METHODOLODY

As mentioned in Chapters 1 and 2, the bulk of the molecular structure of any betacyanin pigment is constituted by its chromophore/aglycone. Pigment destruction occurs at the immonium moiety and is accompanied by a red to yellow transition in colour. Hence, pigment/molecular stability during an investigation may be monitored by following the accompanying color (hue) transitions. Given that pigment extracts from cacti sources consist largely of betacyanins[44], the choice of a Minolta CM 3500d spectrophotometer, as the principle instrument of analysis was well suited. This instrument was able to provide hue (H) value(s) for sample solutions under analysis. Each H value, with the exception of H=0^{o} and 360^{o}, defined a unique loci on the Munsell color space (Fig 5.7). A common locus could be defined by H values of 0^{o} and 360^{O}C

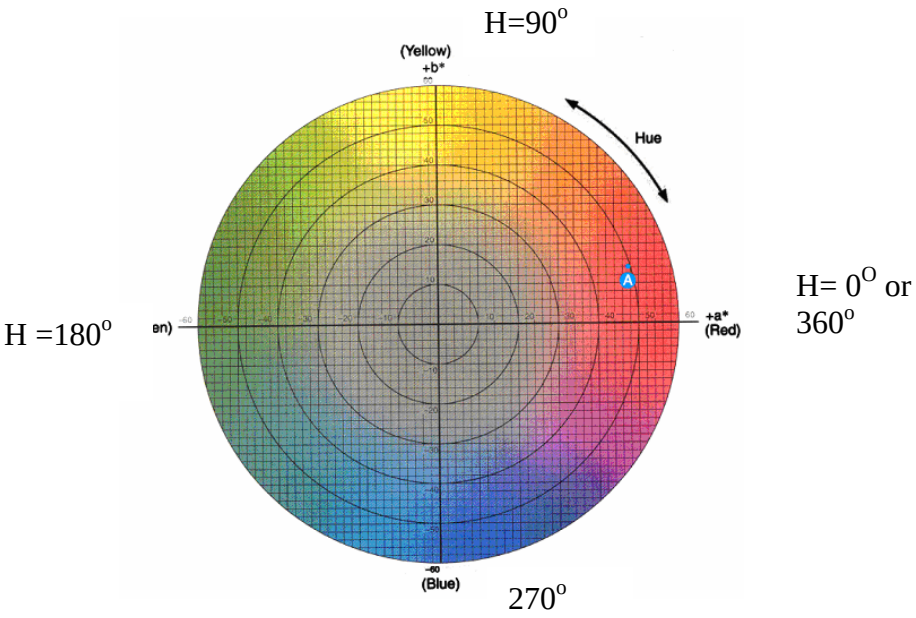


Fig 5.7 Munsell Color Space.

This point has to be kept in mind during measurements of changes in H values. The equations [7] and [8] on the following page, were formulated and employed throughout the course of the study for calculating percentage changes in H values.

$$\Delta H = \frac{H_t + (360^o - H_o)}{H_o} \times 100$$

$$= \frac{(H_t - H_o) + 360^o}{H_o} \times 100 \qquad \textbf{[7]}$$

For $180^o > H_t > 0^o$

$$\Delta H = \frac{H_t - H_o}{H_o} \times 100$$

$$= (\frac{H_t}{H_o} - 1) \times 100 \qquad \textbf{[8]}$$

For $180^o < H_t < 360^o$

H_o: Hue value at time =o

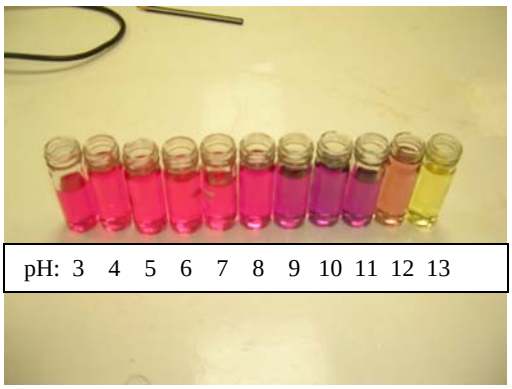
H_t: Hue value at time = t

Units of t: mins (for temperature studies) or days (for comparison of shelf life)

As the Munsell color space is radial in design (fig 5.7), changes in hue could occur in either a clockwise or an anti-clockwise fashion. Betacyanin degradations are accompanied by red to yellow transitions which corresponded to a clockwise change on the color space. Hence, changes in this direction/fashion were designated as positive (+) changes while those occurring in the opposite direction will be designated

as negative (-) changes accordingly. It must be emphasized however that the designations (+) and (-) indicated only the direction of change. In addition, it should be noted that changes in hue were non-punctuated but gradual. As such, it was not possible to define the exact hue values at which color transitions would occur. Visual correlation was often required. A correlation chart exists in the standard Munsell color space itself (Fig 5.7). Alternatively, Fig 5.8 could also serve as a form of visual correlation.

The sample solutions indicated in Fig 5.8 were generated by doping 0.01% (w/v) BetX solutions with 1M NaOH (aq) or HCl (aq) to generate solutions with integer pH values ranging from 3 to 13. Differences in hue might be ascribed to the varying extents of pigment destruction in response to differences in the concentration of hydroxide ions in the indicated solutions[18]. As it was assumed that betacyanin degradations could be based upon similar mechanisms, Fig 5.8 would constitute a reliable correlation yardstick. Based on the visual appearances of the solutions shown in fig 5.8, it might be assumed that visually significant changes in hue, as regards loss of red/pinkish hues, occur at *c.a.* $\Delta H > 4\%$ (pH 9).



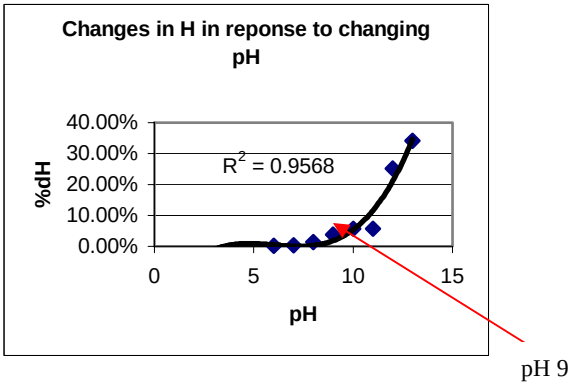


Fig 5.8 (a) **Fig 5.8 (b)**

Fig 5.8(a) 0.01% (w/v) BetX solutions with integer pH values ranging from (left to right) 3 to 13. Note that onset of a discernable loss of the orginal red-pinkish hue first occurs at pH 9. Fig 5.6(b) Graphical representation of fig 5.8 (a)

5.4 COMPARISON OF SHELF-LIFE

In this work, three extraction methods namely, extraction protocols I, II and III were investigated. As mentioned in section 5.3, performance factors for which shelf-life is an important component, are extremely crucial where the suitability of the extraction protocols are concerned.

Solutions of commercial beet juice powder (0.5-1.0%, w/v) have been known to lose their red hues after two weeks[1] when stored at room temperature. The final solution is commonly, yellow brown in appearance. In addition, due to the aqueous nature of the solution, microbial (mould) growth is not uncommon. Therefore, for the purpose of this study, the apparent shelf life of a given extract of betacyanin might be defined as the time, in terms of days, taken for a freshly prepared betacyanin solution to lose its original red-pink appearance. Thus, cessation of shelf life could be marked by the sample solution acquiring a yellowish brown appearance and/or the visible presence of microbial (mould) growth whichever occurs earlier.

This assumption was fairly reliable as shelf-life studies using commercial beet juice powder constituted into solutions containing 0.5% of solids gave results that are consistent with those reported in existing literature[1]. Sample solutions retained red-pink hues for a week before turning yellowish-brown when stored away from light and at a temperature of 28 ^{o}C. Samples refrigerated at 2 ^{o}C turned yellowish brown after 2 weeks of storage.

Solutions prepared using extraction protocol I acquired a yellowish brown appearance just after three days even when protected from light and refrigerated at 2^{o}C. The difference between the original hue of the solution and the hue of the final

solution was greater than 20%. The highly perishable nature of the betacyanins in these extracts might be related to the experimental procedures in extraction protocol I. The initial extract obtained from the overnight stirring/extraction of macerated fruit peel in deionized water at a temperature of 25 ^{o}C was highly viscous and gelatinous. Filtration through ordinary Whatman^{TM} filter paper proved to be an exceedingly arduous task as such. Consequently, glass wool was employed in place of Whatman^{TM} filter paper. However, as the porosity of glass wool was several folds higher, colloidal particles including enzymes capable of pigment degradation could have leeched into the filtrate leading subsequently to the observed pigment degradation.

Betacyanin extracts obtained using extraction protocols II and III underwent marginal changes of less than ±3% in hue values when stored at 25 ^{o}C for a month even in the presence of light. Visually, the final solutions were of a red-pink appearance. The solutions of such extracts, when refrigerated at 2 ^{o}C, were able to retain red-pink appearances for up to 3 months. Interestingly, cessation of shelf life in this instance was marked not by a loss of hue but by the appearance of microbial (mould) growth. Hence, such samples might be capable of potentially longer shelf lives if appropriate preservatives were added.

The reasons(s) behind the observed differences in the shelf lives of betacyanin extracts derived from extraction protocols II and III and that of commercial beet juice powder was not immediately clear. The longer shelf lives demonstrated by betacyanin solutions prepared using extraction protocols II and III, could have been the result of

an absence of betaxanthins as demonstrated for a number of extracts derived from cactaceae sources.

In summary, based on a comparison of the shelf lives of the pigment extracts (Fig 5.9) derived from various extraction methods and/or sources, it is observed that BetX solutions prepared using protocol II and III are capable of much longer shelf lives. It may thus be surmised at this junction, that both protocols would represent suitable/potential extraction methods.

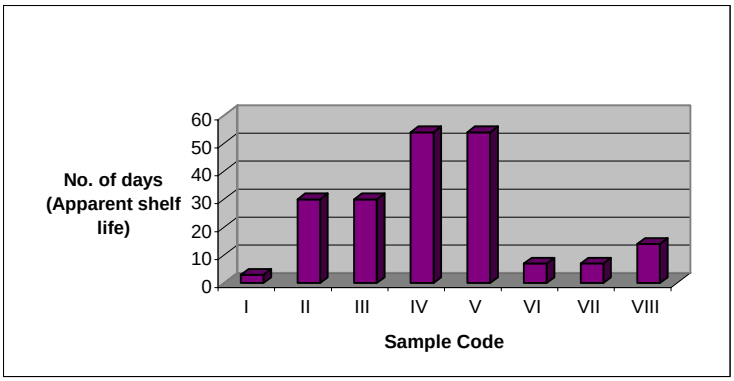


Fig 5.9. Apparent shelf life of variously prepared betacyanin extracts.
I: Pigment extracts prepared using protocol I. Stored at 2 ^{o}C
II: Pigment extracts prepared using protocol II. Stored under ambient conditions.
III: Pigment extracts prepared using protocol III. Stored under ambient conditions
IV: Pigment extracts prepared using protocol II. Stored at 2^{o}C
V: Pigment extracts prepared using protocol III. Stored at 2^{o}C
VI: Beet juice concentrate. Stored at ambient temperature in the dark.
VII: Beet juice concentrate. Stored at ambient temperature under room lighting.
VIII: Beet juice concentrate. Stored at 2^{o}C

5.5 TEMPERATURE STUDIES

Susceptibility to elevated temperatures remains as a major obstacle where the widespread application of betacyanins as natural food colorants is concerned[18]. The degradation cascade mentioned earlier is accelerated during exposure to elevated temperatures. The benefits offered by common stabilizers such as ascorbic acid is very much limited as demonstrated in figure 5.5 due to the far greater reactivity of the

immonium functional group in betacyanins. It is the intention of this study to compare the individual (hue) stability of the variously prepared extracts, in response to various duration of exposure to elevated temperatures. Temperatures of 50 ^{o}C, 70 ^{o}C and 90 ^{o}C and exposure times stretching from zero to thirty minutes were selected as experimental parameters as such conditions are frequently encountered in food processing in general. Sample solutions were adjusted to various pH values (3 to 7) characteristic of common foods, using phosphoric acid and aqueous ammonia prior to the actual investigation as part of the attempts to mimic an actual cooking process.

5.5.1 HUE STABILITY AT 50 ^{o}C

All of the sample solutions employed in this study were able to retain their original red pink appearances even when exposed to a pH range of 3-7 and a temperature of 50 ^{o}C for up to 30 minutes. As illustrated by the graphs in figures 5.10 to 5.14, sample solutions underwent essentially negligible hue transitions of less than ±1.50% relative to their original hue values. As the temperature conditions exacted upon the sample solutions in this particular study may be regarded as being akin to simmering, it might be concluded that the respective betacyanin solutions can function as natural food colorants in foods that involve simmering in their preparation or cooking process.

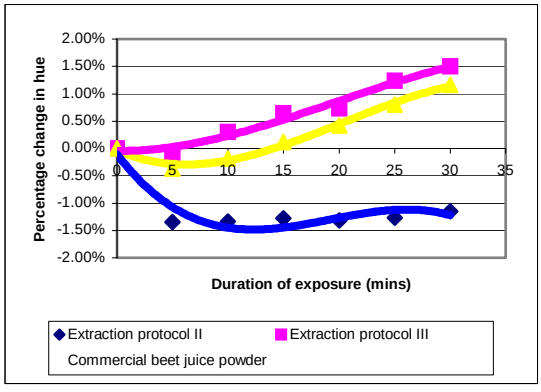


Fig 5.10 Changes in hue of sample solutions at pH 3 in response to increasing duration of exposure to a temperature of 50 ^{o}C.

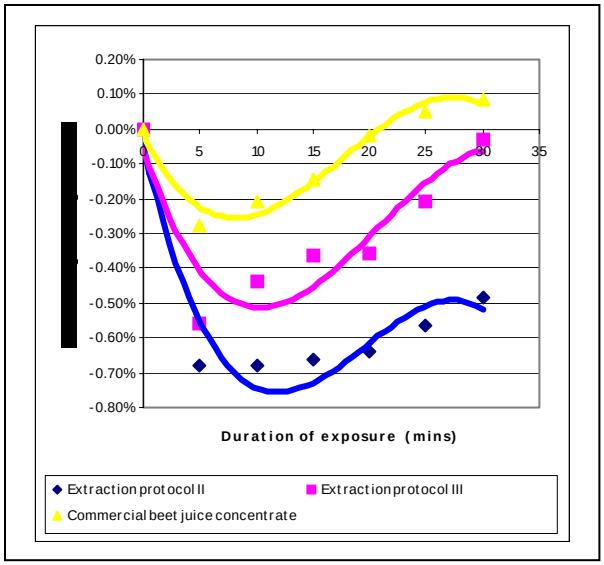


Fig 5.11 Changes in hue of sample solutions at pH 4 in response to increasing duration of exposure to a temperature of 50 ^{o}C.

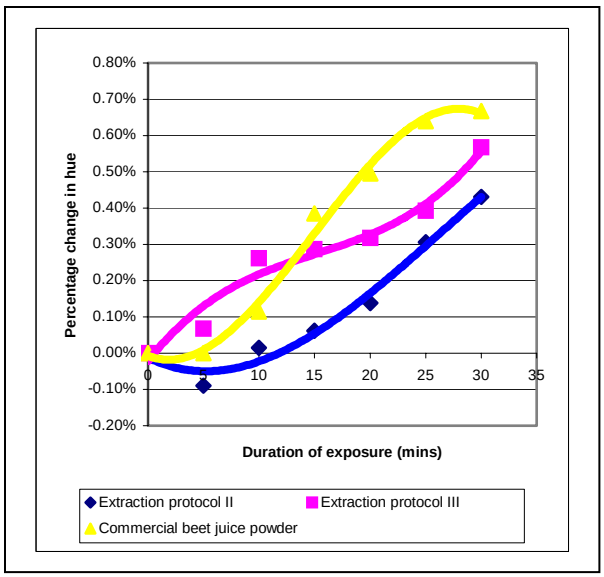


Fig 5.12 Changes in hue of sample solutions at pH 5 in response to increasing duration of exposure to a temperature of 50 ^{o}C.

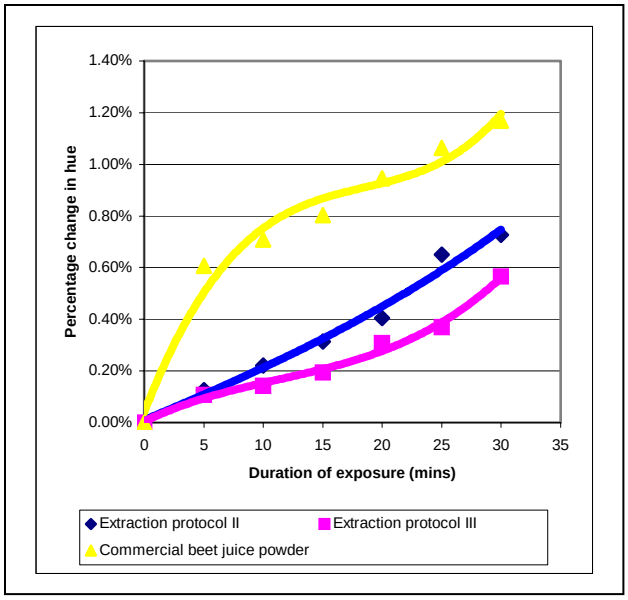


Fig 5.13 Changes in hue of sample solutions at pH 6 in response to increasing duration of exposure to a temperature of 50 ^{o}C.

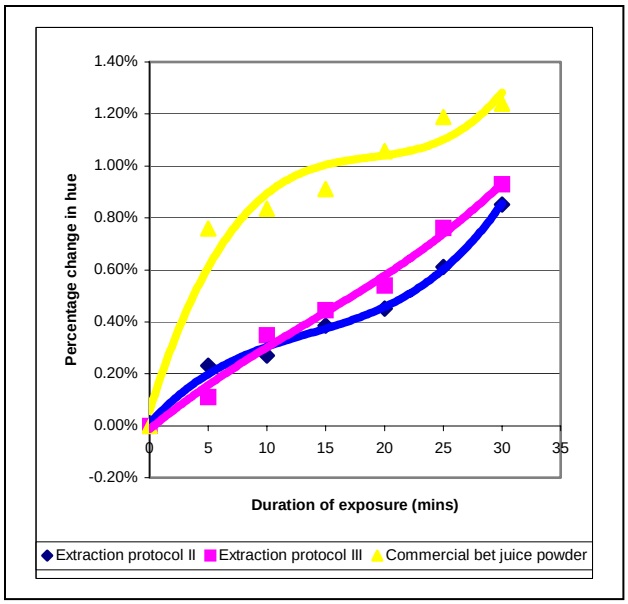


Fig 5.14 Changes in hue of sample solutions at pH 7 in response to increasing duration of exposure to a temperature of 50 ^{o}C.

5.5.2 HUE STABILITY AT 70 ^{O}C

All of the sample solutions, regardless of their pH were generally able to retain their original hue/visual appearances for up to 20 minutes when exposed to temperatures of 70 ^{o}C. The rate of change in hue after 20 minutes is significantly accelerated as illustrated by Figures 5.15 to 5.19. Specifically, this is indicated by the formation of visibly steeper graphical profiles after 20 minutes. Essentially, sample solutions adopted yellowish orange appearances (total percentage change in hue is about 15%) after 25 minutes of exposure to a temperature of 70 ^{o}C. Therefore, processing of the various pigment solutions as natural food colorants at such temperatures must be kept short, in order to minimize the extent of pigment degradation. Interestingly, extracts based on commercial beet juice powder were found to be more susceptible to degradation during exposure to elevated temperatures.

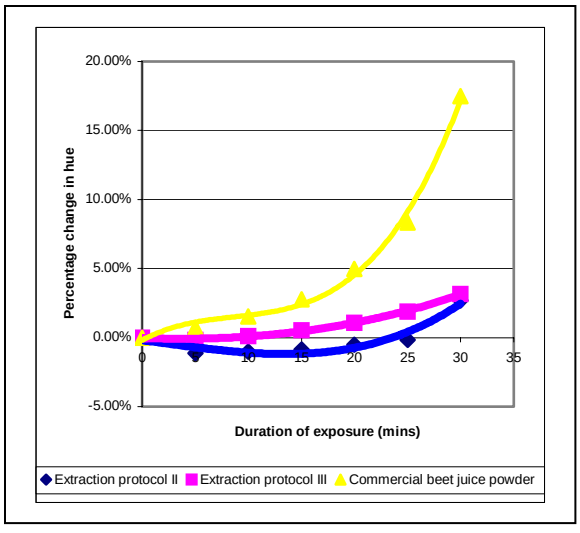


Fig 5.15 Changes in hue of sample solutions at pH 3 in response to increasing duration of exposure to a temperature of 70 ^{o}C.

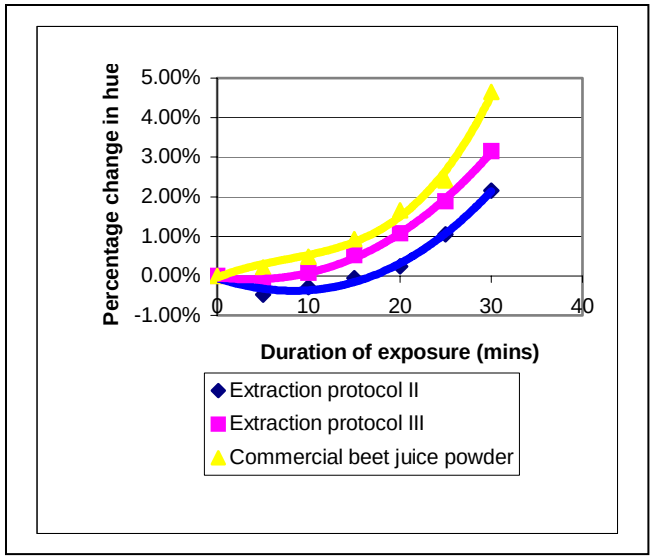


Fig 5.16 Changes in hue of sample solutions at pH 4 in response to increasing duration of exposure to a temperature of 70 ^{o}C.

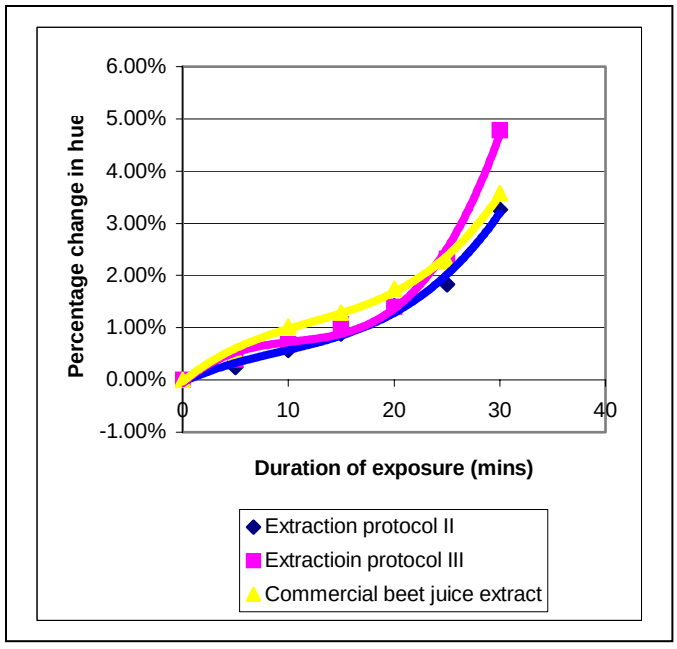


Fig 5.17 Changes in hue of sample solutions at pH 5 in response to increasing duration of exposure to a temperature of 70 ^{o}C.

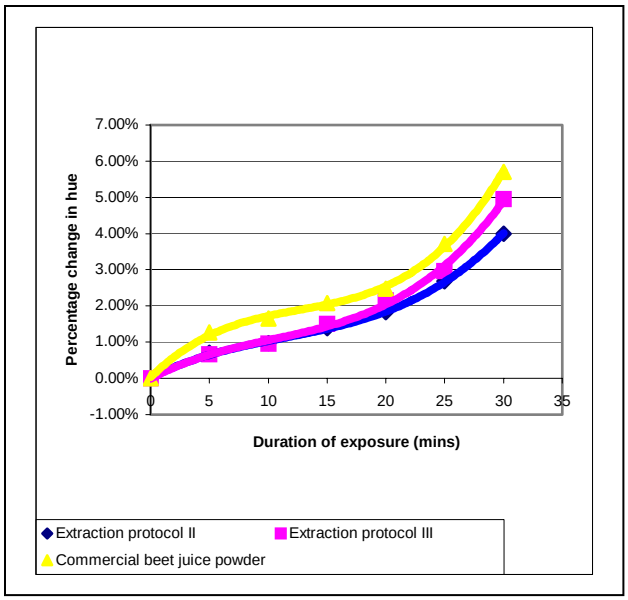


Fig 5.18 Changes in hue of sample solutions at pH 6 in response to increasing duration of exposure to a temperature of 70 ^{o}C.

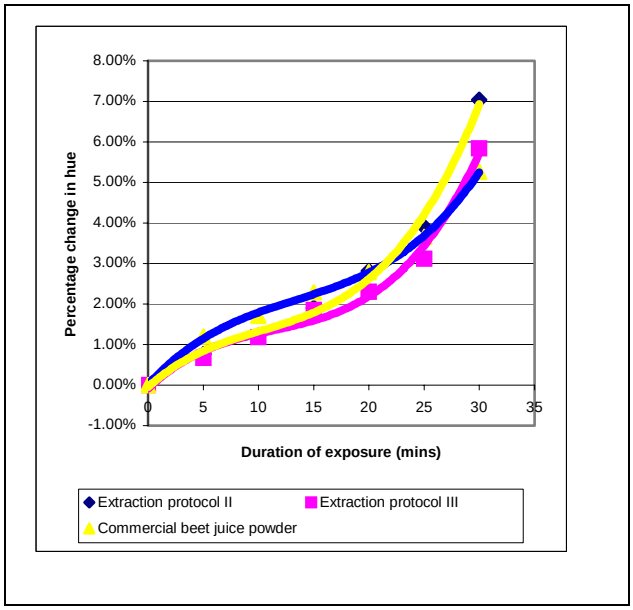


Fig 5.19 Changes in hue of sample solutions at pH 7 in response to increasing duration of exposure to a temperature of 70 ^{o}C.

5.5.3 HUE STABILITY AT 90 ^{O}C

Sample solutions lost their original hues readily when exposed to elevated temperature of 90 ^{o}C. Changes to the original hue values become visually discernable just after 5 minutes as illustrated by the graphical plots in figures 5.20 to 5.24. This is in contrast to samples exposed to a lower temperature of 70 ^{o}C where visually discernable loses in hue occurred only after 25 minutes of exposure. The percentage change in hue after 30 minutes of exposure to 90 ^{o}C can be as high as 30%. Thus, it would be reasonable to suggest that aqueous betacyanin extracts/solutions are not suited for foods for which processing, involves such temperature conditions.

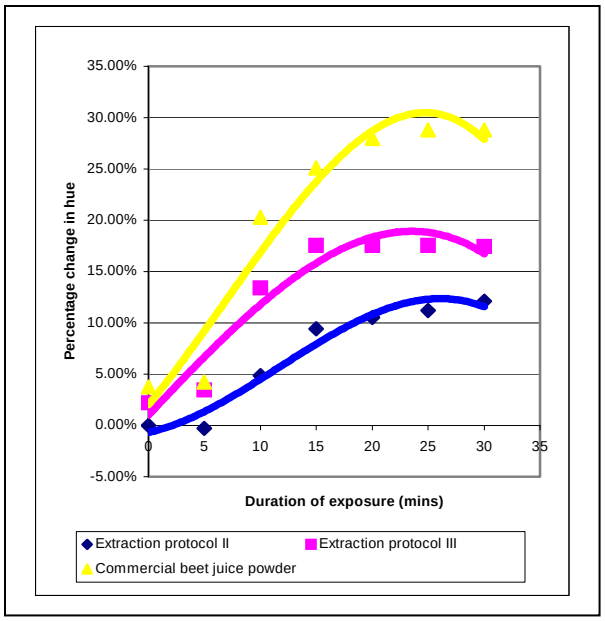


Fig 5.20 Changes in hue of sample solutions at pH 3 in response to increasing duration of exposure to a temperature of 90 ^{o}C.

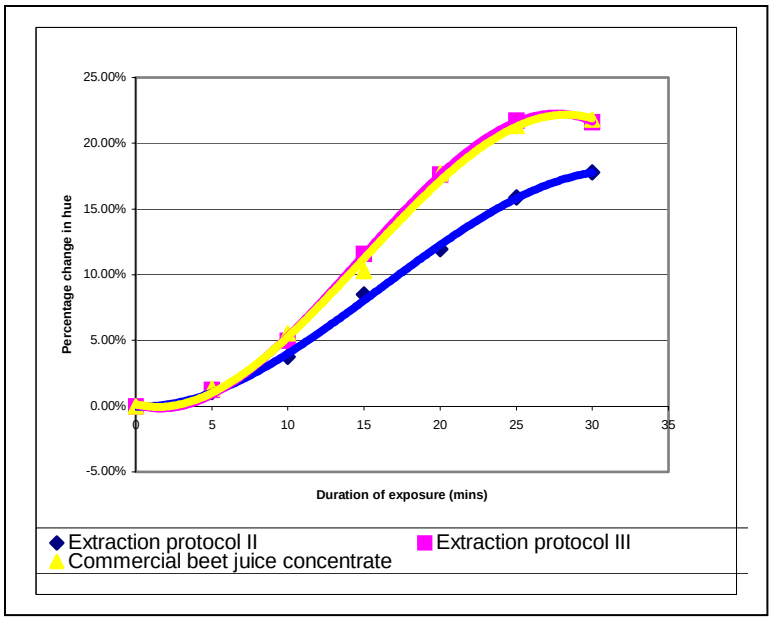


Fig 5.21 Changes in hue of sample solutions at pH 4 in response to increasing duration of exposure to a temperature of 90^{o}C.

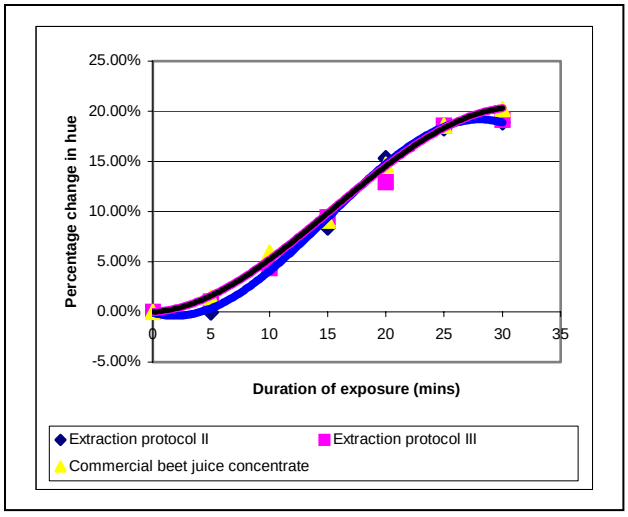


Fig 5.22 Changes in hue of sample solutions at pH 5 in response to increasing duration of exposure to a temperature of 90^{o}C.

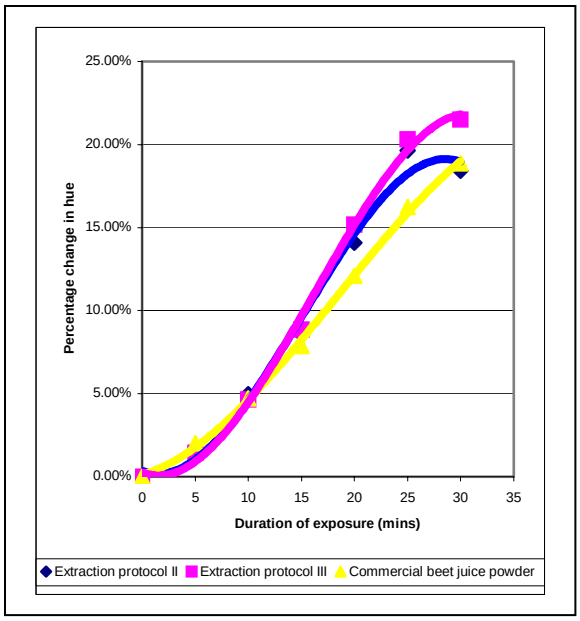


Fig 5.23 Changes in hue of sample solutions at pH 6 in response to increasing duration of exposure to a temperature of 90^{o}C.

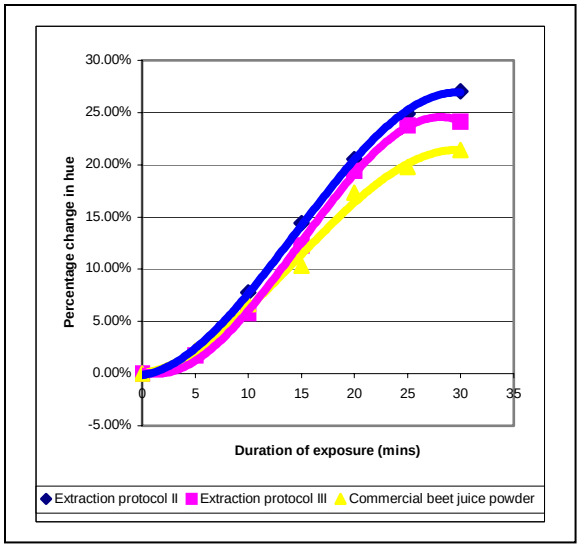


Fig 5.24 Changes in hue of sample solutions at pH 7 in response to increasing duration of exposure to a temperature of 90^{o}C.

5. CONCLUSION

6. CONCLUSION

The results obtained indicated that there is definitely, a potential for the development of natural food colorants from the fruit peel of Dragon fruit (*Hylocereus undatus)*. Most notably, pigments extracts derived from such sources were capable of much longer shelf life than that of beet juice concentrate even in the absence of refrigeration and when exposed to room lighting. In addition, a cost advantage would be inherent from the fact that pigments could be derived from food wastes. Generally, it was possible to derive up to 2500ml of 0.01% BetX (w/v) from *c.a.*600g of fruit peel (6 fruits).

Nonetheless, whilst betacyanins from *H.undatus* are capable of longer shelf lives compared with those of beet root, the scope of application remains the same i.e. confined to food products that are acidic or mildly acidic in nature and with minimized exposure (<15mins) to elevated temperatures of no more than 70 ^{o}C. The means for broadening the application scope of betanidin pigments still remains elusive.

Toxicity studies and the identification of appropriate preservatives for the extracts would bring this aspect of the work to a more satisfactory conclusion.

In general, it may be concluded that the fruit peels of Dragon fruit does possess immense potential for the development of natural food colorants. Further endeavors would probably revolve about the biological applications of the pigments. Two promising areas have been identified as discussed in the next chapter.

7. SUGGESTIONS FOR FUTURE WORK

7.1 BETANIDIN AS A POSSIBLE VEHICLE FOR DRUG DELIVERY

Betanin and its aglycone, betanidin, are essentially organic cations as a consequence of the presence of an immonium functional group[10]. Hence, betanidin and its betacyanin derivatives exhibit salt-like properties in spite of the fact that they are actually organic molecules[14]. Most notably, this family of compounds exhibits good solubility in water and aqueous media but not in common organic solvents such as dichloromethane, chloroform, tetrahydrofuran and alcohols. This, coupled with the susceptibility of the immonium moiety to hydrolysis would suggest that this family of compounds could be of poor oral bioavailability with most of them hydrolysed in the stomach or excreted in the urine[46]. A clinical study conducted by Rina *et al* (2001)[30] however, demonstrated otherwise.

Betanin bioavailability in human volunteers was demonstrated in the above mentioned study by Rina *et al* (2001)in which volunteers consumed 300ml of red beet juice containing 120mg of betanin. Only 0.5 to 0.9% of ingested betanin were calculated to have been excreted in urine. Hence, it might be concluded that the structural integrity of betacyanins was not adversely affected in the gastrointestinal tract and that a significant proportion of the ingested pigment could be successfully absorbed across the intestinal walls.

Such physicochemical characteristics suggest that specially designed/modified betacyanin analogues, in which the catechol-bound (naturally-occurring) sugar unit has been strategically substituted with selected bioactive sugars or their analogues e.g. imino[37] and amino acid[52] sugars, could be potential pro-drugs. This is especially so as the importance of sugars increases with the advent of rational-design drug

discovery methodology and glycomics[37, 52]. This hypothesis was conjectured in the knowledge that the betanidin moiety would allow for the solubilization of the bioactive sugar in aqueous media (if it was not already soluble) as well as facilitate subsequent absorption – commonly observed features of a successful drug delivery process[46].

Nonetheless, the successful development of a betanidin-based drug delivery molecular system is challenged by the lack of suitable ligation methods[55], both chemical and enzymatic, that are able to bind the bioactive sugars with betanidin. In addition, there is the additional requirement that the betanidin-sugar linkage thus formed has to be amendable to cleavage by selected liver enzymes so that "unmasking" of the pro-drug is possible[46].

Promising linkages would take the form of α-glycosidic or ester linkages. α-glycosidic linkages are attractive due to the increasing availability of glycosyl transferase enzymes with broad substrate tolerance and capable of highly chemoselective glycoside bond formation[56]. In addition, liver hydrolases could be utilized for the "unmasking" of the pro-drug[46]. Such linkages however, are disadvantaged by the need for an anomeric centre in the sugar-based drug[37]. This is however, to the best of our knowledge,an uncommon feature in such candidate molecules[37, 52].

In this respect, ester linkages are more attractive since the drug candidate needs only to possess a carboxylic acid functional group - a common functionality in drug candidates, that can be converted into an acid chloride[10, 46, 52]. Subsequent reaction

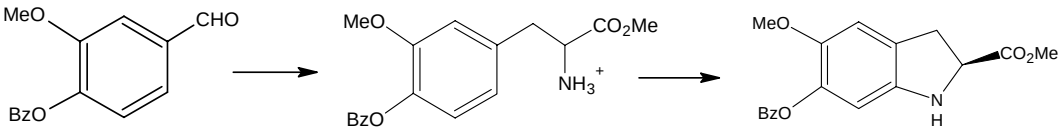
with betanidin's catechol moiety yields an ester linkage[10] that could potentially be amendable to "unmasking" by liver esterases[46].

Hence, there is much room for future research to be conducted in this area.

7.2 TOTAL SYNTHESIS OF BETANIN

As discussed in chapter 2.3, one of the greatest impediments in studies concerned with the potential biological and pharmaceutical applications of betanin, is the absence of an appropriate synthetic strategy. Synthetic routes when available, would allow for structure activity studies, an indispensable effort where attempts to employ the basic betanin structure as a molecular scaffold for assembling drugs are concerned. Successful attempts of this nature have not been reported to date.

Nonetheless, a proposal has been put forth by Tyman[53]. It was envisioned that the starting material consisting of O-benzoylvanillin would be asymmetrically converted to L-β-(4-O-benzoyl-3-methoxyphenyl) alanine methyl ester and thence to the 4-O-benzoyl-3-O-methyl derivative of L-cyclodopa methyl ester (Fig 7.1). The inherent challenge here is to identify practically viable reaction conditions and reagents.



O-Benzoylvanillin

L-beta-(4-O-benzoyl-3-methoxyphenyl) alanine methyl ester

Fig 7.1 Proposed synthetic strategy for the synthesis of the 4-O-benzoyl-3-O-methyl derivative of L-cyclodopa methyl ester.

Subsequent reaction with the semicarbazone of betalamic acid diester, available via the synthetic route reported by Shapiro *et al* (1977)[54] would afford the 5-O-benzoyl,6-methyl ether of betanidine/isobetanidine dimethyl esters (Fig 7.2). Reaction conditions for this step could be adapted from those reported in the same work by Shapiro and his co-workers.

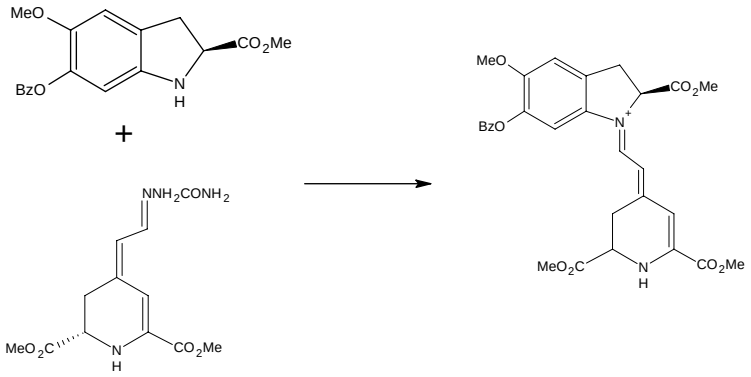


Fig 7.2 Synthesis of 5-O-benzoyl,6-methyl ether of betanidine/isobetanidine

Selective demethylation with aluminium triiodide and reaction with 2,3,4,5,-tetra-O-acetyl-α-D-glucosylbromide ($BrAc_4G$) followed by mild hydrolysis/deprotection would in principle, lead to betanin/isobetanin[53] (Fig 7.3). Again however, there is a need to identify viable reaction conditions.

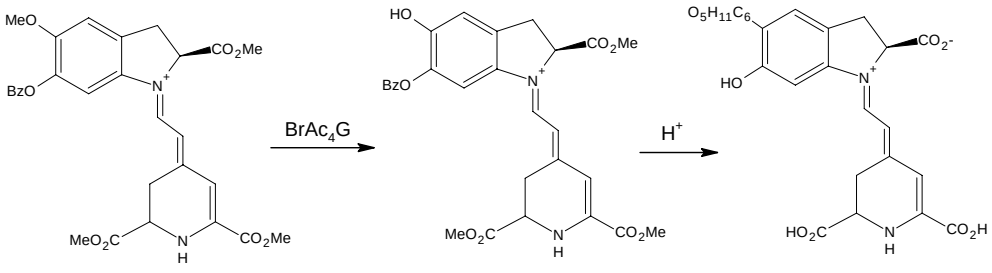


Fig 7.3 Generation of betanin/isobetanin

8. BIBLOGRAPHY

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1. Henry B.S. in *Natural Food Colorants*, 2nd Edition. Edited by G.A.F Henry and J.D. Houghton. 1997, Blackie & Professional. Great Britain.

2. Taylor R.J. *Food Additives*. 1984, John Wiley & Sons. New York.

3. DuBose C.N., Cardello A.V. and Maller O.J. *Food Science.* 1980, 45, 1393-1399.

4. Pangborn R.M. *American Journal of Psychology*. 1960, 43, 229-238.

5. John M.deMan in *Principles of Food Chemistry*. 3rd Edition. 1999, Aspen Publishers Inc. USA.

6. Wissgott U. and Bortlik K. *Trends in Food Science & Technology*. 1996, 7, 298-302.

7. J.M. Jones in *Food Safety*. 2nd Printing. 1993, Eagen Press. USA.

8. F.J Francis in *Colorants*. 1999, Eagen Press. USA.

9. G.R. Gibson and C.M. Williams in *Functional foods: concept to product.* 2000, CRC Press. Boca Raton, F.L.

10. J. March in *Advanced Organic Chemistry*. 5th Edition. 1992, N.Y. Wiley. USA.

11. Duxbury D.D. *Food Process.* 1990. 63-70.

12. F.Delgado-Vagras, Jimenez A.R. and Parades-Lopez. *Critical Reviews in Food Science and Nutrition*. 2000, 40(3), 173-289.

13. Stuppner H. and Egger R. *Journal of Chromatography A.* 1996, 735, 409-413.

14. F.Delgado-Vagras and Parades-Lopez in *Natural Colorants for Food and Nutraceutical Uses.* 2002. CRC Press. Boca Raton F.L.

15. Loughrey K. in *Natural Food Colorants. Science and Technology*. Edited by G.J. Lauro and F.J. Francis. 2000. Marcel Dekker Inc. USA.

16. *Precise Color Communication: Color control from perception to instrumentation.* Technical publication. Minolta Co. Ltd.

17. Von Elbe J.H. and Goldman I.L. in *Natural Food Colorants. Science and Technology*. Edited by G.J Lauro and F.J. Francis. 2000. Marcel Dekker Inc. USA.

18. Strack D., Vogt T. and Schliemann W. *Phytochemistry*. 2003, 62(3), 247-269.

19. Wyler H. and Dreiding A.S. *Helvetica Chimica Acta*. 1957, 40, 191.

20. (a) Robinson G.M. and Robinson R. *Journal of Chemical Society*. 1932, 1439.
(b) Robinson G.M. and Robinson R. *Journal of Chemical Society*. 1933, 25

23. Mabry T.J. *Journal of Natural Products*. 2001, 64, 1596-1604.

24. Mabry T.J., Wyler H., Parikh I. and Dreiding A.S. *Tetrahedron*. 1967, 23(7), 3111-27.

25. Schliemann W., Kobayashi N. and Strack D. *Plant Physiology*. 1999, 119, 1217-1232.

26. Piatelli M. in *Betalains. In Chemistry and Biochemistry of Plant Pigments Vol 1*. Edited by Goodwin T.W. 2nd Edition. 1976. Academic Press. London.

27. J.F. Morton in *Fruits of Warm Climate*. 1987. Media Incorporated. USA.

28. Bokern M., Heuer S. and Strack D. *Botannica Acta*. 1992, 105, 146-151.

29. Schwartz S.J. *Diss. Abst. International.* 1983, 43(11), 3524-3525.

30. Schwartz S.J., Von Elbe J.H., Pariza M.W., Goldsworthy T. and Pitot H.C. *Food and Chemical Toxicology.* 1983, 21(5), 531-535.

31. Kanner J., Harel S. and Granit R. *Journal of Agricultural and Food Chemistry*. 2001, 49, 5178-5185.

32. Nilsson T. *Lantbruk-Shoegskolans Analytica.* 1970, 56, 179-219.

33. M. Piatelli in *The Biochemistry of Plants: A Comprehensive Treatise. Vol 17. Secondary Plant Products.* Edited by E.E. Conn. 1981. Academic Press. New York.

34. Kolb E., Huang E., Janzowski C., Vetter A. and Eisenbrand G. *Food and Chemical Toxicology*. 1997, 35(2), 219-224.

35. Koplas-Lane L.M. and Warthesen J.J.. *Journal of Food Science*. 1995, 60(4), 773-776.

36. Ershov A.V., Lukin W.J., and Bazan N.G. *Journal of Biological Chemistry*. 1996, 271(45), 28458-28462.

37. G.Legler in *Imino sugars as glycosidase inhibitors: Nojirimycin and beyond*. Edited by A.E. Sttz. 1999. Wiley-VCH. Weinhein. New York.

38. Stintzing F.C., Scieber A. and Carle R. *Food Chemistry*. 2002, 77(1), 101-106.

39. Piatelli M. and Minale L.. *Phytochemistry*. 1964, 3, 307-311.

40. Wybraniec S., Platzner I. and Geresh S. *Phytochemistry*. 2001, 58, 1209-1212.

41. Stintzing F.C, Scieber A. and Carle R. *Food Chemistry*. 2002, 77(4), 517.

42. Wybraneic S., Mizrahi Y. *Journal of Agricultural and Food Chemistry*. 2002, 50(21), 6086-6089.

43. Strack D., Wogt D. and Schliemann W. *Phytochemistry*. 2003, 62(3), 247-269.

44. Reynoso R., Garcia F.A., Morales D. and E.J. de Mejia. *Journal of Agricultural and Food Chemistry*. 1997, 45, 2884-2889.

45. Kobayashi N., Schmidt J., Nimtz M., Wray V. and Schliemann W. *Phytochemistry*. 2000, 34, 419-426.

46. G.L. Patrick in *An Introduction to Medicinal Chemistry*. 2001. Oxford University Press. New York.

47. *Vietnam News*. August 11, 2001. *http://vietnamnews.vnagency.com.vn/2001-08/10/stories/11.htm*

48. T.H. Lim. *Honours thesis: Studies of color pigments from Hylocereus undatus*. 2002. National University of Singapore. Singapore.

49. *The Sale of Food Act. Chapter 283*. Penal Code. Republic of Singapore.

50. Locey C.L. and Guzinski G.A. in *Natural Food Colorants. Science and Technology*. Edited by G.J. Lauro and F.J. Francis. 2000. Marcel Dekker Inc. USA.

51. *Methods in Biotechnology: Natural Products Isolation*. Edited by R.J.P. Cannel. 1998. Humana Press. New Jersey. USA.

52. Gruner S., Locardi E., Lohof E. and Kessler H. *Chemical Reviews*. 2002, 102(2), 491-514.

53. Tyman J.H.P in *Studies in Natural Products Chemistry. Vol 20.* Edited by Atta-Ur-Rahman. 1998. Elsevier Science. New York.

54. Buchi G., Fliri H. and Shapiro R. *Journal of Organic Chemistry*. 1977, 42, 2192-4

9. APPENDIX

Appendix 1.1 : Temperature studies for pigment extracts prepared using protocol II

Temp: 50°C

pH: 3

Duration of Exposure/mins	H1	H2	H3	Average
0	356.41	356.49	356.42	356.44
5	351.75	351.68	351.52	351.65
10	351.64	351.67	351.73	351.68
15	351.87	351.92	351.91	351.90
20	351.77	351.66	351.79	351.74
25	352.01	351.86	351.92	351.93
30	352.31	352.37	352.34	352.34

pH: 4

Duration of Exposure/mins	H1	H2	H3	Average
0	356.23	356.28	356.33	356.28
5	353.83	353.92	353.80	353.85
10	353.81	353.86	353.91	353.86
15	353.88	353.96	353.95	353.93
20	353.88	354.10	354.02	354.00
25	354.23	354.26	354.29	354.26
30	354.57	354.57	354.51	354.55

pH: 5

Duration of Exposure/mins	H1	H2	H3	Average
0	355.54	355.65	355.49	355.56
5	353.30	355.23	355.19	355.24
10	356.64	355.57	355.62	355.61
15	355.75	355.78	355.81	355.78
20	356.03	356.05	356.07	356.05
25	356.57	356.70	356.68	356.65
30	357.13	357.11	357.03	357.09

pH: 6

Duration of Exposure/mins	H1	H2	H3	Average
0	356.31	356.26	356.24	356.27
5	356.63	356.78	356.75	356.72
10	357.09	357.06	357.03	357.06
15	357.36	357.44	357.37	357.39
20	357.72	357.76	357.65	357.71
25	355.54	355.64	355.59	355.59
30	355.86	355.83	355.89	355.86

Temperature studies for pigment extracts prepared using protocol II cont'd

pH: 7

Duration of Exposure/mins	H1	H2	H3	Average
0	354.75	354.75	354.66	354.72
5	352.47	355.59	355.56	355.54
10	355.64	355.69	355.71	355.68
15	356.12	356.05	356.10	356.09
20	356.24	356.37	356.35	356.32
25	356.84	356.93	356.90	356.89
30	357.65	357.76	357.81	357.74

Appendix 1.1: Temperature studies for pigment extracts prepared using protocol II

Temp: 70°C

pH : 3

Duration of Exposure/mins	H1	H2	H3	Average
0	356.41	356.45	356.46	356.44
5	352.34	352.43	352.46	352.41
10	352.77	352.70	352.75	352.74
15	353.41	353.32	353.35	353.36
20	354.60	354.61	354.56	354.59
25	355.85	355.96	355.86	355.89
30	6.01	5.92	5.95	5.96

pH: 4

Duration of Exposure/mins	H1	H2	H3	Average
0	356.25	356.28	356.31	356.28
5	354.61	354.55	354.62	354.59
10	355.24	355.32	355.25	355.27
15	356.13	356.09	355.99	356.07
20	357.15	357.08	357.13	357.12
25	0.01	0.02	0.01	0.01
30	3.98	3.95	3.98	3.97

pH: 5

Duration of Exposure/mins	H1	H2	H3	Average
0	355.54	355.58	355.56	355.56
5	356.45	356.42	356.39	356.42
10	357.56	357.63	357.58	357.59
15	358.69	358.75	358.75	358.73
20	0.53	0.54	0.67	0.58
25	2.05	2.09	2.04	2.06
30	7.19	7.14	7.12	7.15

pH: 6

Duration of Exposure/mins	H1	H2	H3	Average
0	356.27	356.31	356.23	356.27
5	358.81	358.83	358.76	358.80
10	359.72	359.72	359.81	359.75
15	1.23	1.31	1.27	1.27
20	2.77	2.70	2.75	2.74
25	5.85	5.79	5.85	5.83
30	10.52	10.52	10.55	10.53

Temperature studies for pigment extracts prepared using protocol II cont'd

pH: 7

Duration of Exposure/mins	H1	H2	H3	Average
0	354.71	354.74	354.71	354.72
5	357.39	357.50	357.46	357.45
10	359.19	359.02	359.00	359.07
15	1.45	1.53	1.55	1.51
20	4.68	4.72	4.73	4.71
25	8.59	8.60	8.52	8.57
30	19.71	19.68	19.65	19.68

Appendix 1.1: Temperature studies for pigment extracts prepared using protocol II

Temp: 90°C

pH :3

Duration of Exposure/mins	H1	H2	H3	Average
0	356.42	356.40	356.50	356.44
5	355.49	355.47	355.39	355.45
10	13.72	13.88	13.77	13.79
15	29.96	30.03	29.92	29.97
20	34.01	33.99	33.94	33.98
25	36.37	36.33	36.32	36.34
30	39.61	39.55	39.61	39.59

pH:4

Duration of Exposure/mins	H1	H2	H3	Average
0	356.25	356.31	356.38	356.28
5	0.07	0.19	0.11	0.10
10	9.60	9.53	9.64	9.59
15	26.49	26.49	26.43	26.47
20	38.80	38.72	38.82	38.78
25	52.83	52.75	52.76	52.78
30	59.54	89.57	29.62	59.57

pH:5

Duration of Exposure/mins	H1	H2	H3	Average
0	355.61	355.53	355.54	355.56
5	355.47	355.42	355.49	355.46
10	13.02	13.14	13.14	13.10
15	25.58	25.54	25.38	25.50
20	49.97	49.95	50.02	49.98
25	60.59	60.73	60.63	60.65
30	62.65	62.66	62.61	62.64

pH:6

Duration of Exposure/mins	H1	H2	H3	Average
0	356.28	356.21	356.32	356.27
5	2.31	2.32	2.36	2.33
10	13.99	14.07	14.00	14.02
15	26.79	26.79	26.88	26.82
20	46.47	46.46	46.57	46.50
25	66.19	66.22	66.25	66.22
30	61.85	61.88	6.91	61.88

Temperature studies for pigment extracts prepared using protocol II cont'd

pH:7

Duration of Exposure/mins	H1	H2	H3	Average
0	354.71	354.75	354.70	354.72
5	2.06	2.04	2.11	2.07
10	22.24	22.25	22.20	22.23
15	45.93	45.80	45.88	45.87
20	67.69	67.72	67.66	67.69
25	83.10	83.02	83.03	83.05
30	90.70	90.75	90.68	90.71

Appendix 1.2 : Temperature studies for pigment extracts prepared using protocol III

Temp: 50°C

pH: 3

Duration of Exposure/mins	H1	H2	H3	Average
0	352.19	352.24	352.17	352.20
5	351.83	351.86	351.83	351.84
10	353.28	353.28	353.22	353.26
15	354.49	354.43	354.46	354.46
20	354.82	354.70	354.79	354.46
25	356.46	356.42	356.56	356.48
30	357.41	357.43	357.48	357.44

pH: 4

Duration of Exposure/mins	H1	H2	H3	Average
0	352.16	352.09	352.11	352.12
5	350.14	350.20	350.11	350.15
10	350.55	350.58	350.61	350.58
15	350.85	350.83	350.87	350.85
20	350.90	350.82	350.89	350.87
25	351.38	351.41	351.35	351.38
30	352.05	352.01	352.00	352.02

pH: 5

Duration of Exposure/mins	H1	H2	H3	Average
0	352.10	352.06	352.08	352.08
5	352.34	352.35	352.27	352.32
10	353.02	353.01	352.97	353.00
15	353.14	353.08	353.05	353.09
20	353.23	353.16	353.21	353.20
25	353.49	353.45	353.44	353.46
30	354.08	354.10	354.06	354.08

pH: 6

Duration of Exposure/mins	H1	H2	H3	Average
0	351.58	351.60	351.53	351.57
5	351.90	351.98	351.97	351.95
10	352.10	352.04	352.07	352.07
15	352.24	352.23	352.28	352.25
20	352.63	352.65	352.67	352.65
25	352.90	352.84	352.87	352.87
30	353.55	353.54	353.59	353.56

Temperature studies for pigment extracts prepared using protocol II cont'd

pH: 7

Duration of Exposure/mins	H1	H2	H3	Average
0	350.87	351.01	350.97	350.95
5	351.33	351.31	351.38	351.34
10	352.14	352.16	352.21	352.17
15	352.53	352.53	352.47	352.51
20	352.81	352.88	352.83	352.84
25	353.59	353.63	353.64	353.62
30	354.19	354.20	354.25	354.21

Appendix 1.2: Temperature studies for pigment extracts prepared using protocol III

Temp: 70°C

pH : 3

Duration of Exposure/mins	H1	H2	H3	Average
0	352.30	352.18	352.12	352.20
5	358.91	358.87	358.86	358.88
10	357.88	357.90	357.86	357.88
15	1.16	1.14	1.21	1.17
20	356.33	356.31	356.36	356.33
25	11.29	11.28	11.24	11.27
30	23.16	23.17	23.24	23.19

pH: 4

Duration of Exposure/mins	H1	H2	H3	Average
0	352.09	352.12	352.14	352.12
5	351.61	351.55	351.55	351.57
10	352.41	352.46	352.39	352.42
15	353.94	353.96	354.01	353.97
20	355.90	355.95	355.88	355.91
25	358.73	358.82	358.70	358.75
30	3.20	3.17	3.26	3.21

pH: 5

Duration of Exposure/mins	H1	H2	H3	Average
0	352.10	352.00	352.14	352.08
5	353.46	353.50	353.42	353.46
10	354.49	354.47	354.54	354.50
15	355.86	355.79	355.84	355.83
20	357.05	356.98	357.06	357.03
25	0.22	0.27	0.23	0.24
30	8.92	8.95	8.86	8.91

pH: 6

Duration of Exposure/mins	H1	H2	H3	Average
0	351.61	351.56	351.54	351.57
5	353.90	353.87	353.93	353.90
10	354.92	355.01	354.98	354.97
15	356.84	356.89	356.82	356.85
20	359.15	359.21	359.15	359.17
25	2.00	1.99	2.04	2.01
30	9.02	9.00	9.07	9.03

Temperature studies for pigment extracts prepared using protocol II cont'd

pH: 7

Duration of Exposure/mins	H1	H2	H3	Average
0	350.98	350.91	350.96	350.95
5	353.31	353.27	353.32	353.30
10	355.01	355.13	355.13	355.09
15	357.42	357.42	357.51	357.45
20	359.05	358.98	359.03	359.02
25	1.93	1.87	1.87	1.89
30	11.51	11.45	11.46	11.47

Appendix 1.2: Temperature studies for pigment extracts prepared using protocol III

Temp: 90°C

pH :3

Duration of Exposure/mins	H1	H2	H3	Average
0	352.17	352.22	352.21	352.20
5	4.45	4.52	4.44	4.47
10	39.51	39.53	39.58	39.54
15	54.03	54.12	54.12	54.09
20	54.04	52.98	53.42	53.48
25	54.08	54.11	53.99	54.06
30	53.66	53.75	53.67	53.69

pH:4

Duration of Exposure/mins	H1	H2	H3	Average
0	352.10	352.11	352.15	352.12
5	356.45	356.39	356.45	356.43
10	9.64	9.65	9.70	9.66
15	32.87	32.95	32.94	32.92
20	54.09	54.08	54.13	54.10
25	68.54	68.60	68.57	68.57
30	68.16	68.19	68.13	68.16

pH:5

Duration of Exposure/mins	H1	H2	H3	Average
0	352.10	352.04	352.09	352.08
5	355.76	355.66	355.72	355.68
10	7.38	7.39	7.46	7.41
15	25.23	25.28	25.27	25.26
20	37.57	37.60	37.54	37.57
25	57.41	57.38	57.32	57.37
30	59.52	59.64	59.55	59.57

pH:6

Duration of Exposure/mins	H1	H2	H3	Average
0	351.62	351.54	351.55	351.57
5	356.67	356.71	356.63	356.57
10	7.90	7.92	7.97	7.93
15	22.76	22.69	22.68	22.71
20	44.90	44.84	44.93	44.89
25	63.10	62.97	63.02	63.03
30	67.24	67.20	67.16	67.20

Temperature studies for pigment extracts prepared using protocol II cont'd

pH:7

Duration of Exposure/mins	H1	H2	H3	Average
0	351.02	350.95	350.88	350.95
5	356.05	356.96	357.05	357.02
10	11.22	11.27	11.29	11.26
15	33.94	33.98	33.87	33.93
20	59.06	59.13	59.05	59.08
25	74.25	74.26	74.33	74.28
30	75.60	75.61	75.53	75.58

Appendix 1.3 : Temperature studies for commercial beet juice extracts

Temp: 50°C

pH: 3

Duration of Exposure/mins	H1	H2	H3	Average
0	344.23	347.54	348.99	346.92
5	346.90	350.55	339.44	345.63
10	344.14	347.84	346.86	346.28
15	346.94	348.63	346.45	347.34
20	347.66	348.57	348.88	348.37
25	349.43	350.08	349.56	349.69
30	350.75	351.31	350.76	350.94

pH: 4

Duration of Exposure/mins	H1	H2	H3	Average
0	346.14	348.02	347.11	347.09
5	346.42	347.21	344.76	346.13
10	345.4	347	346.68	346.36
15	346.9	346.73	346.14	346.59
20	348.01	347.21	345.87	347.03
25	347.15	347.73	346.93	347.27
30	347.04	347.47	347.63	347.28

pH: 5

Duration of Exposure/mins	H1	H2	H3	Average
0	347.55	347.41	346.88	347.28
5	347.87	347.33	346.64	347.28
10	347.47	347.44	348.13	347.68
15	349.03	348.36	348.47	348.62
20	348.91	349.07	349.02	349.00
25	349.71	349.18	349.61	349.5
30	349.82	349.60	349.38	349.6

pH: 6

Duration of Exposure/mins	H1	H2	H3	Average
0	345.44	345.92	343.38	345.88
5	347.60	348.25	348.09	347.98
10	348.93	347.89	348.17	348.33
15	346.23	349.54	350.25	348.66
20	349.17	348.12	350.16	349.15
25	349.33	350.48	348.87	349.56
30	350.60	350.01	349.15	349.92

Temperature studies for comercial beet juice extracts

pH: 7

Duration of Exposure/mins	H1	H2	H3	Average
0	344.9	334.73	355.07	344.99
5	347.92	347.46	347.45	347.61
10	348.08	347.5	348.03	347.87
15	347.96	348.17	348.26	348.13
20	348.52	348.38	349.02	348.64
25	348.97	349.03	349.27	349.09
30	349.25	349.18	349.38	349.27

Appendix 1.3: Temperature studies for commercial beet juice extracts

Temp: 70°C

pH : 3

Duration of Exposure/mins	H1	H2	H3	Average
0	346.95	347.01	346.80	346.92
5	349.42	349.34	349.41	349.39
10	352.14	352.35	352.32	352.27
15	356.85	356.16	356.43	356.48
20	4.15	4.00	4.15	4.10
25	15.67	15.82	15.79	15.76
30	47.62	47.68	47.38	47.56

pH: 4

Duration of Exposure/mins	H1	H2	H3	Average
0	347.11	347.02	347.14	347.09
5	347.97	347.91	347.67	347.85
10	348.82	348.79	348.73	348.78
15	350.53	350.15	350.28	350.32
20	352.81	352.92	352.79	352.84
25	355.51	355.47	355.43	355.47
30	3.17	3.25	3.27	3.23

pH: 5

Duration of Exposure/mins	H1	H2	H3	Average
0	347.25	347.28	347.31	347.28
5	349.06	349.07	349.14	349.09
10	350.82	350.87	350.80	350.83
15	351.82	351.77	351.63	351.74
20	353.36	353.42	353.27	353.35
25	355.20	355.10	355.21	355.17
30	359.67	359.72	359.71	359.70

pH: 6

Duration of Exposure/mins	H1	H2	H3	Average
0	345.86	345.91	345.87	345.88
5	350.31	350.26	350.27	350.28
10	351.59	351.53	351.62	351.58
15	353.02	353.09	353.10	353.07
20	353.98	354.39	355.01	354.46
25	358.69	358.84	358.66	358.73
30	5.55	5.65	5.63	5.61

Temperature studies for pigment extracts prepared using protocol II cont'd

pH: 7

Duration of Exposure/mins	H1	H2	H3	Average
0	344.92	344.97	345.08	344.99
5	349.15	349.08	349.10	349.11
10	350.87	350.89	350.91	350.89
15	352.92	352.88	352.93	352.91
20	354.57	354.64	354.65	354.62
25	357.67	357.43	357.73	357.61
30	3.06	3.16	3.20	3.14

Appendix 1.3: Temperature studies for commercial beet juice extracts

Temp: 90°C

pH :3

Duration of Exposure/mins	H1	H2	H3	Average
0	346.91	346.88	346.97	346.92
5	1.62	1.58	1.57	1.59
10	57.24	57.31	57.26	57.27
15	73.98	74.05	74.00	74.01
20	84.06	83.96	83.92	83.98
25	86.73	86.77	86.90	86.80
30	86.81	86.83	86.76	86.80

pH:4

Duration of Exposure/mins	H1	H2	H3	Average
0	347.11	347.06	347.10	347.09
5	351.65	351.72	351.64	351.67
10	6.25	6.31	6.31	6.29
15	22.81	22.81	22.75	22.79
20	48.57	48.62	48.55	48.58
25	60.97	61.12	61.03	61.04
30	62.65	62.74	62.68	62.69

pH:5

Duration of Exposure/mins	H1	H2	H3	Average
0	347.31	347.22	347.31	347.28
5	352.15	352.17	352.22	352.18
10	7.74	7.64	7.72	7.70
15	18.92	18.89	18.83	18.88
20	38.29	38.33	38.22	38.28
25	51.75	51.80	51.63	51.73
30	57.42	57.33	57.45	57.40

pH:6

Duration of Exposure/mins	H1	H2	H3	Average
0	345.82	345.90	345.92	345.88
5	352.91	352.83	352.90	352.88
10	2.19	2.19	2.13	2.17
15	13.05	13.14	13.08	13.09
20	27.66	27.72	27.75	27.71
25	42.01	42.08	42.12	42.07
30	51.06	51.13	51.11	51.10

Temperature studies for commercial beet juice extracts
cont'd

pH:7

Duration of Exposure/mins	H1	H2	H3	Average
0	344.88	344.96	345.13	344.99
5	352.59	352.48	352.64	352.57
10	8.00	7.98	8.02	8.00
15	20.59	20.67	20.57	20.61
20	44.68	44.71	44.71	44.70
25	53.31	53.19	53.28	53.26
30	58.91	58.95	58.93	58.93